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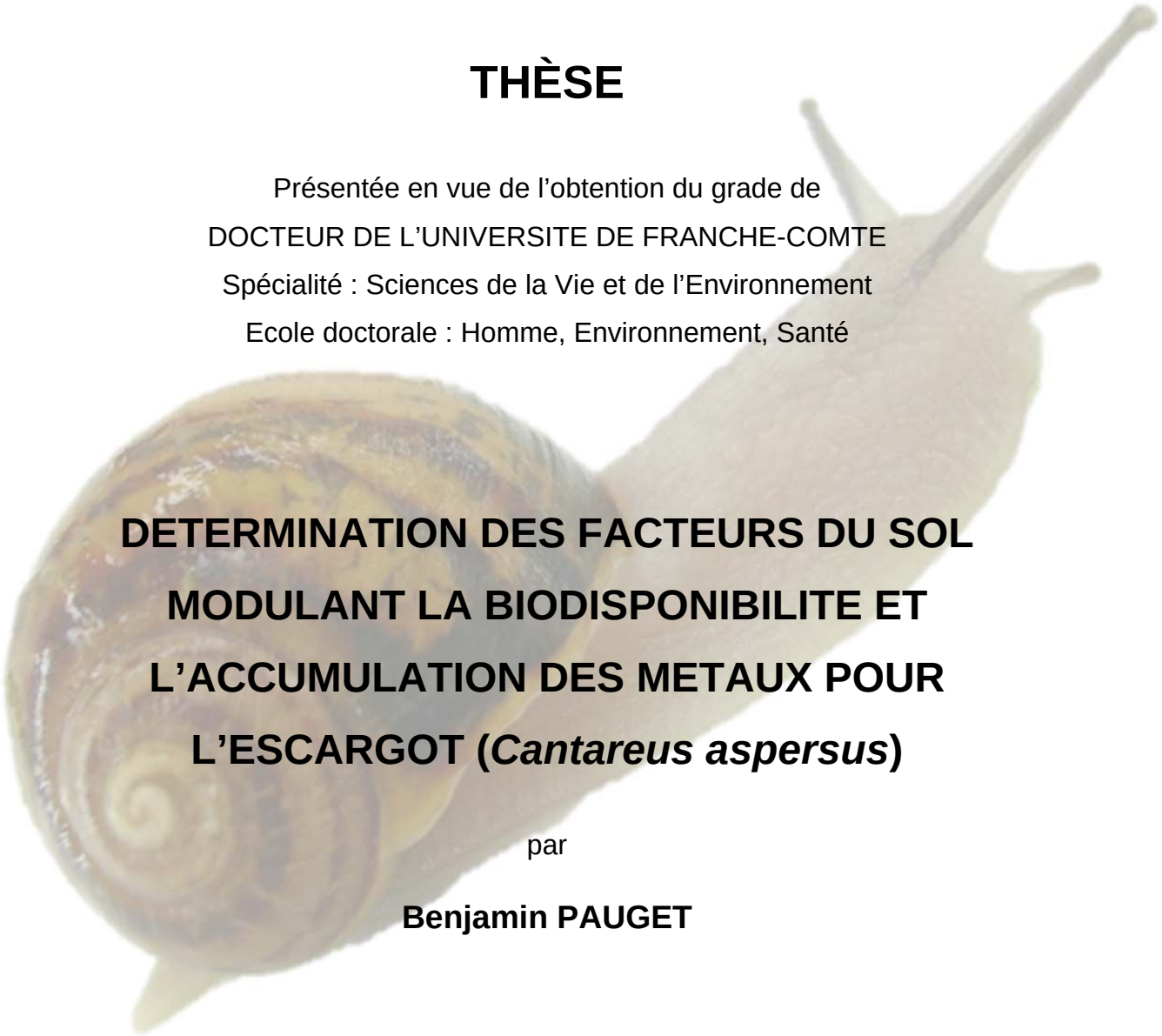

DETERMINATION DES FACTEURS DU SOL MODULANT LA BIODISPONIBILITE ET L'ACCUMULATION DES METAUX POUR L'ESCARGOT (*Cantareus aspersus*)

par

Benjamin PAUGET

Soutenue le 12 Juillet 2012 devant le jury composé de :

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Avant propos

Cette thèse traite de l'influence des caractéristiques physico-chimiques des sols et des sources de contamination sur le transfert et la biodisponibilité des métaux du sol chez l'escargot *Cantareus aspersus*, espèce inféodée au sol et vivant à l'interface sol-plante-air. Une part importante de la thèse repose sur une approche ***in situ*** visant à appréhender l'importance des caractéristiques des sols dans l'estimation de la biodisponibilité des métaux pour l'escargot. Les études conduites **en laboratoire** ont permis quant à elles de renseigner plus précisément l'influence des paramètres du sol et des sources de contaminations sur la biodisponibilité des métaux.

Ce manuscrit présente l'aboutissement de 3,5 années d'études réalisées dans le cadre d'une thèse financée par une allocation du ministère de l'enseignement supérieur et de la recherche. Elle s'est déroulée au laboratoire Chrono-Environnement à Besançon. Ce travail a bénéficié d'autre part du contexte scientifique et du financement du programme ADEME Bioindicateur Phase 2 (Bio2, http://ecobiosoil.univ-rennes1.fr/ADEME-Bioindicateur/).

Le manuscrit est organisé en quatre parties :

La première (A) présente les contaminants étudiés, à savoir les métaux (leurs origines et mobilité au sein des sols), les approches actuelles d'évaluation des risques liée aux métaux présents dans les sols et les méthodes d'évaluation de leur biodisponibilité. Cette première partie présente également l'organisme bioindicateur utilisé au cours de ce travail : l'escargot *Cantareus aspersus.* Elle expose également les méthodes et les outils biologiques et mathématiques originaux développés au cours de mon master (l'étude ayant servi de base de travail pour cette thèse est présentée en Annexe 1) pour l'étude cinétique des transferts. Sur la base de cet état des connaissances et des lacunes identifiées, les objectifs de la thèse sont définis en fin de cette première partie.

La seconde (B) est consacrée à l'étude *in situ* de l'accumulation et de la biodisponibilité des métaux pour l'escargot et à l'influence des paramètres des sols sur ces deux paramètres. Cette partie est divisée en 3 chapitres :

Le premier concerne la présentation des sites d'études du programme Bioindicateur 2 qui ont servi pour l'exposition des escargots *in situ*.

Le second est consacré à une étude statique de la biodisponibilité (accumulation après 28 jours d'exposition) et aux paramètres des sols la modulant. Les objectifs sont de poser les bases d'un référentiel des concentrations d'escargots exposés sur un large choix de sites et de proposer un nouvel outil d'évaluation des transferts de métaux (SET).

Le troisième concerne une étude cinétique sur trois sites industriels de la biodisponibilité des métaux et des paramètres des sols qui l'influence.

La troisième partie (C) s'articule en deux chapitres consacrés aux expérimentations en laboratoire :

La premier chapitre de cette partie se focalise sur la quantification de la contribution des sources sol et plante à la biodisponibilité des métaux pour l'escargot et vise à déterminer si le pH et/ou le taux de matière organique font varier cette contribution.

Le second chapitre vise à établir des équations prédictives de la biodisponibilité en considérant le sol comme unique source de contamination et à déterminer si la biodisponibilité des métaux pour

l'escargot peut être modélisée à partir de mesures chimiques de la concentration en métaux disponibles dans les sols.

Enfin, la dernière partie (D) discute de la relation entre les résultats présentés dans les quatre chapitres. Elle fait le bilan de la contribution de cette étude à la recherche en écotoxicologie et restitue l'ensemble de ces apports dans le contexte global de l'évaluation des risques de transfert des métaux. Les perspectives envisagées dans la continuité de ces travaux sont également présentées.

Listes des publications et participation aux congrès

Publication dans des revues à comité de lecture

2009 - de Vaufleury A., Fritsch C., Gimbert F., **Pauget B.**, Coeurdassier M., Crini N., Scheifler R., Utilisation et intérêts des escargots et des micromammifères pour la bioindication de la qualité des sols. Etude et Gestion des Sols 2009 ; 16 : 203-217

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Communication

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2008 - Gimbert F., **Pauget B.**, Coeurdassier M., Scheifler R., Badot P.M. and de Vaufleury A. From environmental availability to bioavailability : soil-type-dependent metal toxicokinetics in snail. 18th Annual Meeting of SETAC Europe, May 25th-29th 2008, Warsaw, Poland.

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2010 - Hedde M., Bispo A., Cluzeau D., Cortet J., Douay F., Galsomies L., Grand C., Houot S., Leyval C., **Pauget B.**, Ruiz-Camacho N., Ulrich E., Vandenbulcke F., de Vaufleury A., Villenave C., Péres G. Programme ADEME - Bioindicateur II : Recherche d'outils de surveillance, de caractérisation et d'évaluation des risques des sols basés sur la faune du sol. Ecologie 2010, Montpellier, France. (Communication orale de M Hedde)

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2011 - **Pauget B.**, Gimbert F., Coeurdassier M., Alaphilippe A., Bispo A., Beguiristain T., Douay F. , Faure O., Galsomies L., Grand C., Hedde M., Hitmi A. , Houot S., Legras M., Peres G., Ulrich E., Vian J.F., de Vaufleury A., Détermination de concentrations internes de référence (CIRef) en éléments traces métalliques de l'escargot Petit Gris sur divers sites français et essais de caractérisation biologique des sites contaminés. Colloque de la SEFA, Versailles, France (Poster)

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2012 - **Pauget B.**, Coeurdassier M., Gimbert F., Pérès G., Alaphilippe A., Beguiristain T., Douay F., Faure O., Hitmi A., Houot S., Legras M., Guernion M., Vian J.-F. and de Vaufleury A. Snail watch: a tool to assess the risk of metal transfer taking into account their bioavailability. SETAC Europe, 22nd annual meeting, May 20th-24th 2012, Berlin, Germany

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« Pollution : les escargots jouent les indics ». 2008. Auteur : Laurianne Geffroy, réalisation : Jean-Pierre Courbatze, Ya+K productions. Reportage diffusé à la Cité des Sciences et de l'Industrie, La Villette, Paris. 2008.

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Liste des abréviations

AFNOR : agence Française de normalisation
Al_{ox} : oxyde d'aluminium
ASPITET : Apport d'une stratification pédologique pour l'interprétation des teneurs en éléments traces
BAF : bioaccumulation factor
BARGE: Bioaccessibility Research Group in Europe
CBR : Critical body residues
CEC : Capacité d'échange cationique
CIRef : concentration interne de référence
C_{org} : carbone organique
DGT : diffusive gradient in thin film
Diss : métaux totaux dissous dans la solution du sol
EA : Environmental availability
EB : Environmental bioavailability
ERA: environmental risk assessment
ERE : Evaluation du risque environnemental
ERI : évaluation du risque individuel
ETM : élément trace métallique
FBA : Facteur de bioaccumulation
Fe_{ox} : oxyde de fer
FIAM : free ion activity model
IEM : interprétation de l'état des milieux
Ion : concentration ionique dans la solution du sol
ISO: international standarisation organisation
MO : Matière organique
MT: Métallothionéine
PEC : Predictive environmental concentration
PNEC : Predictive No Effect concentration
RMQS : réseau de mesures de la qualité des sols
SET : somme des excès de transfert
US EPA: United State environmental protection agency
VTR : valeur toxicologique de référence

Partie A : Introduction

I. Historique

Durant le dernier siècle, l'Europe a connu une phase de forte expansion industrielle accompagnée de l'exploitation de nombreuses ressources non renouvelables. Le manque de préoccupation environnementale de cette époque a conduit au déversement de nombreuses substances polluantes dans l'environnement. Celles qui étaient peu voire non dégradables se sont accumulées dans les sols, les sédiments, les eaux et les organismes (Commission of the European communities, 2006). Même si cette exploitation a permis un fort développement économique, elle a été à l'origine d'une dégradation de notre environnement via une chute de la biodiversité et de la qualité des milieux naturels. Il a été identifié que l'industrialisation, l'agriculture intensive et l'expansion démographique (Figure 1) de ces dernières années ont conduit à un dysfonctionnement des sols se traduisant par une diminution de la biodiversité ainsi que d'une diminution de la dégradation des polluants dans les sols. Parmi le grand nombre de polluants menaçant les sols, les métaux jouent un rôle important (Figure 2) de par leur forte persistance dans l'environnement (Commission of the European communities, 2006; INRA, 2011).

II. Pollution des sols par les éléments trace métalliques (ETM)

II.1. Définition

Les éléments trace métalliques (ETM) font partie de la famille des éléments trace (ET). Ensemble, ils ne représentent que 0,6% du total des éléments. Leur dénomination de trace est due à leur faible concentration qui n'excède généralement pas 1000 $mg.kg^{-1}$ naturellement dans les sols (Alloway, 1995; Baize, 1997). Parmi ces ETM on peut citer le cadmium (Cd), le chrome (Cr), le cuivre (Cu), le plomb (Pb), le zinc (Zn) qui se classent dans la catégorie des métaux et l'arsenic (As) et l'antimoine (Sb) qui se classent parmi les métalloïdes. Une classification des ETM a été proposée par Nieboer et Richardson (Nieboer and Richardson, 1980) qui les scinde en trois classes selon leur affinité avec certains ligands : les ligands oxygénés (classe A), les ligands azotés ou soufrés (classe B) ou pour les deux types de ligands (classe intermédiaire). Cette classification a permis une meilleure compréhension des mécanismes de stockage et d'excrétion des ETM par les organismes.
Les cinq métaux et deux métalloïdes étudiés dans cette thèse appartiennent à la classe intermédiaire.

II.2. Origine

Les ETM présents dans les sols sont pour partie d'origine naturelle, en provenance de la dégradation du matériau parental (Girard et al., 2005), des feux de forêts et des éruptions volcaniques (Garrett, 2000). Les ETM dans les sols peuvent également être d'origine anthropique. En effet, leur utilisation par nos sociétés a augmenté de 300% ces cinquante dernières années entrainant une multiplication par trois des rejets de métaux tels que le Pb ou le Cu (INERIS, 2006). Les principales sources anthropiques de métaux sont l'agriculture avec par exemple l'utilisation de l'arséniate de plomb comme insecticide (Girard et al., 2005 ; INERIS, 2010), la pollution urbaine due au trafic routier et à l'incinération d'ordures (Denison and Silbergeld, 1988) et le rejet dans l'environnement de métaux sous forme d'apports diffus (poussières) ou localisés par rejet direct dans l'environnement (Godin et al., 1985; Baize, 1997; Merian et al., 2004) (Tableau 1).

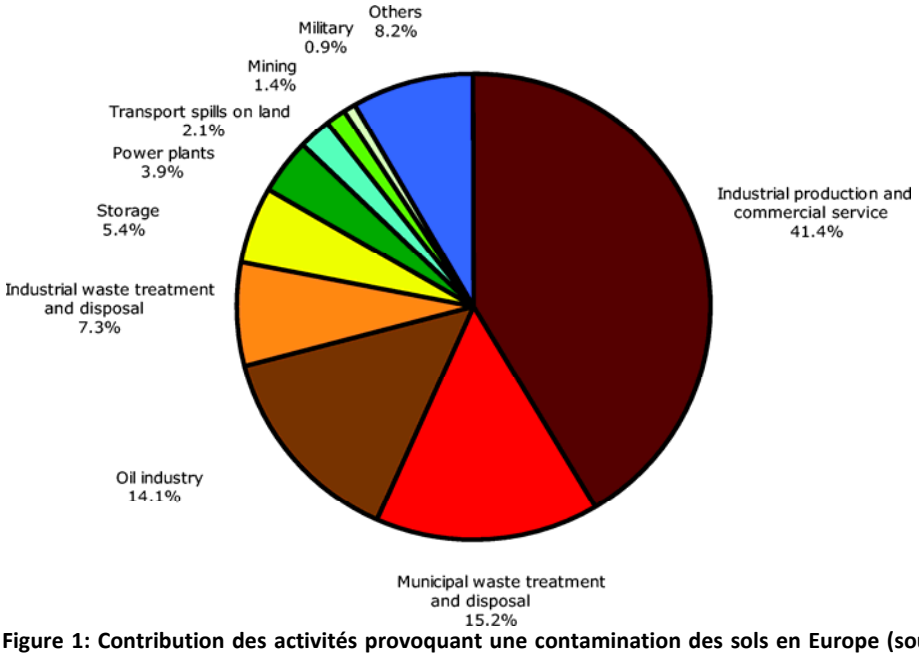


Figure 1: Contribution des activités provoquant une contamination des sols en Europe (source : European Environment Agency, 2006).

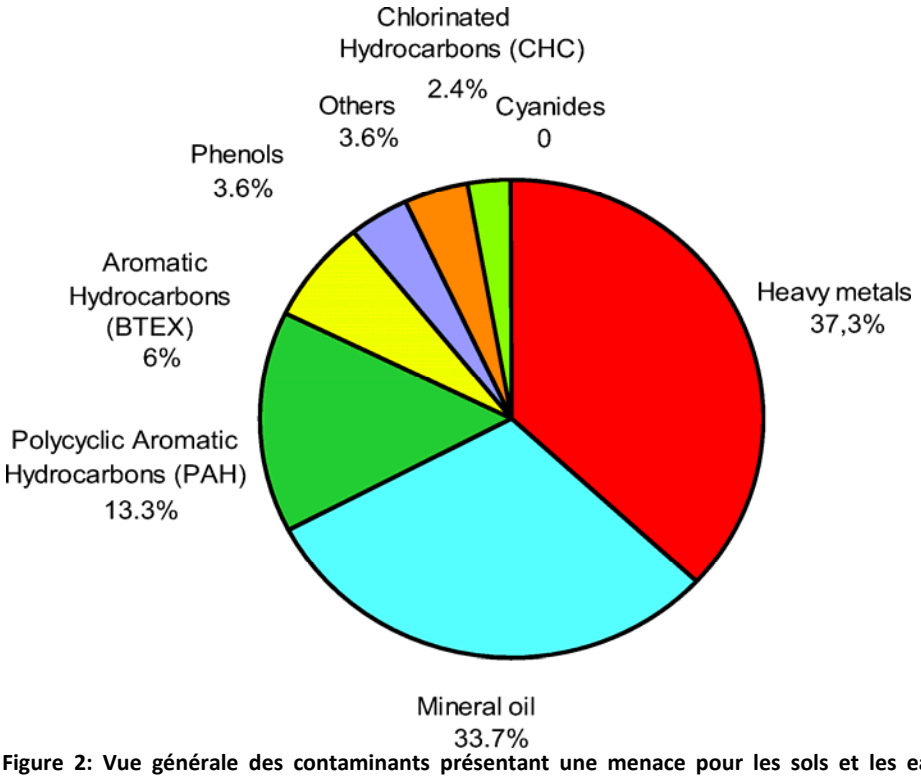


Figure 2: Vue générale des contaminants présentant une menace pour les sols et les eaux souterraines en Europe. (source : European Environment Agency, 2006).

Tableau 1 : Contribution des différentes sources à l'enrichissement moyen annuel des terres émergées en ETM (Feix and Wiart, 1998).

Source	Elément			
	Cd	Pb	Cu	Zn
Total (10^3 t)	20	382	216	760
Déchets agricoles	20%	12%	55%	61%
Déchets urbains	2%	19%	28%	20%
Engrais	37%	10%	1%	1%
Retombées atmosphériques	40%	68%	16%	18%

La préoccupation liée à la présence de ces métaux dans les sols est en grande partie due à leurs effets néfastes tant sur l'environnement que sur la santé humaine. En effet, dans la décision 2455/2001/CE du conseil Européen, trois métaux (Cd, Pb et mercure (Hg)) ont été identifiés comme substances dangereuses prioritaires, l'objectif étant d'arriver à un rejet zéro de ces substances dans les eaux souterraines (INERIS, 2006). De nombreux métaux ont été classés comme cancérigènes ou comme dangereux pour la santé humaine (INERIS, 2006; Huff et al., 2007; Qingdong et al., 2007). Il est donc indispensable de connaitre leur devenir dans l'environnement.

III. Evaluation des risques liés aux ETM dans les sols en Europe et en France

III.1. Historique

Il y a encore quelques dizaines d'années, la pollution des sols était perçue comme la résultante d'accidents ponctuels avec de possibles conséquences sur la santé humaine et l'environnement. Cependant, quelques noirs épisodes environnementaux (Love canal, Minamata…) ont alerté les politiques publiques. Suite à ces événements, un contrôle maximum des risques ainsi qu'un enlèvement ou confinement complet de la pollution fut demandé par les pouvoirs publics. Les Etats-Unis ont ainsi créé le programme Superfund qui vise à identifier les sites de déchets abandonnés potentiellement dangereux. Il permet à l'Environmental Protection Agency (EPA) de dépolluer ces sites ou de contraindre les responsables des pollutions à prendre en charge les opérations de dépollution.

En 1994, un forum commun aux états membres de l'Union Européenne (UE) et l'Agence Européenne de l'Environnement (AEE) se fixait comme objectif de coopérer dans la lutte contre les sites pollués en centralisant les techniques et pratiques pour leur réhabilitation. Lors de leur première réunion à Bonn (Allemagne), la recommandation de la création d'un projet européen sur l'évaluation des risques des sites contaminés s'est traduit par l'action concertée CARACAS (Concerted Action for Risk Assessment for Contaminated Sites in Europe). Ce travail a été centré sur sept domaines thématiques dont celui des méthodes d'évaluation des risques écologiques et celui des comportements et transport des polluants dans les sols.

Aujourd'hui, la pollution des sols n'est plus le seul critère de leur gestion. En effet, les diverses menaces pesant sur la qualité des sols influencent en grande partie la politique de gestion des sites et sols pollués (Commission of the European communities, 2006). Cependant, tous les pays européens n'ont pas développé de législation spécifique en matière de sols contaminés. Par exemple, le Code de l'Environnement et ses articles relatifs à la gestion des installations classées sont les seuls documents en vigueur en France (Guide relatif aux " Modalités de gestion et de réaménagement des sites pollués ", (INERIS, 2007)) alors que par exemple la Belgique s'est dotée d'outils réglementaires spécifiques pour la gestion des sols pollués (décret sur la réhabilitation des sols en 1995, le « décret sol » et « l'ordination sur la gestion des sites pollués en 2004, (Ferguson et al., 2005)). Les Pays-Bas ont quant à eux développé des valeurs d'interventions pour les sols ainsi que

des valeurs cibles (si la concentration dans les sols est inférieure à cette valeur cible alors le sol est adapté à tout type d'usage, (Ferguson et al., 2005)).
Du projet CARACAS sont issus des principes fondamentaux comme le principe du pollueur-payeur, le principe de précaution, l'utilisation de l'évaluation des risques pour l'identification, la hiérarchisation et l'évaluation du besoin d'actions de réhabilitation avec lesquels s'accordent les 16 pays européens partenaires du projet. Cependant, des différences entre les pratiques des différents pays demeurent et l'harmonisation de ces pratiques au sein de l'union européenne telles que l'utilisation de valeurs toxicologiques de référence (VTR) et de valeurs seuil de contamination des sols, la prise en compte des facteurs socio-économiques, parait être indispensable (Hayet et al., 2009).

III.2. Evaluation du risque environnemental (ERE)

Parmi tous les polluants présents dans l'environnement, les ETM posent de nombreux problèmes de santé publique du fait de leur persistance, de leur mobilité et leur toxicité (Commission of the European communities, 2006; INERIS, 2006). Afin d'évaluer l'impact de leur présence dans l'environnement tant sur les écosystèmes que sur l'homme et pour préserver les milieux, des procédures d'évaluation des risques ont été mises en place.
L'ERE est une procédure qui tente de calculer ou d'estimer le risque pour une cible (organisme, un système ou une sub-population) engendré suite à l'exposition à un ou plusieurs agent(s), et en incluant les incertitudes associées (USEPA, 1998; European Commission, 2003; IPCS, 2004; Hayet et al., 2009). Même si les pratiques des pays de l'UE diffèrent (utilisation ou non de valeurs guides, de valeurs seuils…), elles restent globalement assez similaires (Jensen and Pedersen, 2006; Hayet et al., 2009; Smith, 2009). L'objectif de l'évaluation du risque est d'obtenir des données sur les risques qui soient transparentes et scientifiquement fondée pour étayer le processus de prise de décisions. L'ERE peut se faire par une approche substance (analyse des substances prises individuellement) qui consiste à déterminer la relation entre l'exposition prévue et les effets défavorables. Cette approche comporte quatre étapes principales, à savoir (i) l'identification du danger, (ii) l'évaluation de la relation dose-réponse ou la caractérisation du danger, (iii) l'évaluation de l'exposition et (iv) la caractérisation du risque (IPCS, 2004). Concrètement, pour une approche substance, la caractérisation du risque se fait en général par comparaison des PNEC (Predictive No Effect concentration : concentration prévue sans effet) et des PEC (Predictive environmental concentration : concentration prévisible dans l'environnement) dans chacun des compartiments concernés. Si le ratio PEC/PNEC est inférieur à 1 alors la pollution ne présente aucun risque pour les organismes. L'ERE se base donc sur des données expérimentales grâce auxquelles sont déterminées des valeurs seuils comme les valeurs toxicologiques de référence (VTR), qui sont les fondations de l'établissement d'un risque (Fairbrother et al., 2007; Bur et al., 2010).
Cependant, cette méthode concernant une approche substance n'est pas la seule utilisée en ERE. En effet, en fonction du type de pollution, une approche matrice peut être utilisée. Cette approche se base sur la mesure des effets (effets toxicologiques,…) sur les organismes d'une source de contamination complexe multicontaminées (sol, effluent, sédiment…). Plus concrètement, des essais écotoxicologiques sur des gammes de dilution de la matrice sont réalisés. Cette approche permet d'appréhender les interactions ainsi que les transferts de l'ensemble des contaminants aux organismes.
En France, la démarche d'interprétation de l'état des milieux (IEM) est basée sur ces deux approches associées à une approche site spécifique (IEM, 2007). Cet outil, axé sur la santé humaine, vise à déterminer si l'état d'un site est en accord avec l'usage que l'on souhaite en faire grâce à une grille de calcul qui se base sur les concentrations en contaminant dans différentes sources d'exposition (sol, air et alimentation), l'exposition (quantité de contaminant ingérée) et sur les valeurs toxicologiques de référence (VTR) du ou des contaminants étudié(s). De cette grille ressort un quotient de danger (QD, pour les substances à seuil d'effet) ou un excès de risque individuel pour

l'homme (ERI, pour les substances sans seuil d'effet) pour chaque substance étudiée et permet de valider ou non l'usage que l'on souhaite faire du site.
Cependant, cette méthode basée sur un risque théorique n'est pas forcément représentative de la réalité. En effet, la mobilité ainsi que le transfert des métaux sont modulés par de nombreuses variables comme les paramètres des sols (Sterckeman et al., 2004) qui ne sont pas pris en compte dans l'IEM.
Dans le but d'intégrer les facteurs de variation des transferts et d'élargir le nombre d'items considérés dans l'ERE, l'approche écologique peut être utilisée. Elle permet de caractériser les effets d'une pollution sur les communautés et les populations via des mesures de diversité de communautés ou de structure des populations *in situ*. L'approche écologique comporte également des études de biosurveillance qui constituent un bon complément aux approches substance et matrice. Ces études visent à évaluer le potentiel dangereux d'un ou plusieurs polluant(s) en fonction du temps et de l'espace sur des organismes cibles. La biosurveillance met en jeu des bioindicateurs, organismes servant à identifier la présence ou l'effet d'un polluant (Markert, 2007). Ces bioindicateurs sont donc des outils de mesure de la qualité de l'environnement et sont complémentaires aux mesures physico-chimiques (Fairbrother et al., 2007). De plus, la biosurveillance permet de détecter des pollutions quand les capteurs peuvent rester insensibles. Parmi les bioindicateurs, deux catégories peuvent être distinguées (Figure 3) : les bioindicateurs d'accumulation permettant d'évaluer une exposition aux polluants et les bioindicateurs d'effets qui répondent spécifiquement à une pollution mais qui renseignent également sur l'intensité de la réponse induite par cette exposition, la réponse mesurée étant en général proportionnelle à la dose assimilée (Heink and Kowarik, 2010).

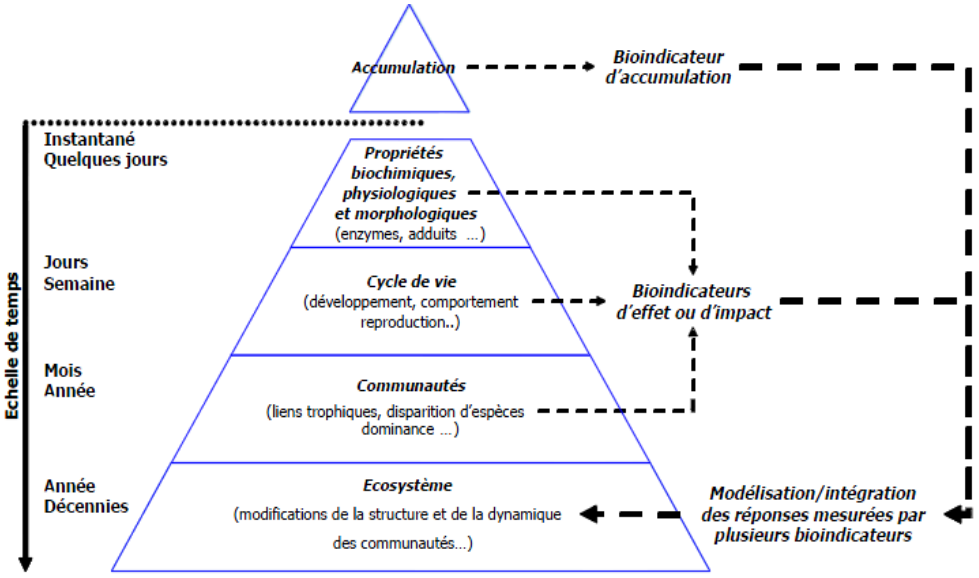


Figure 3 : les différents niveaux de réponses des bioindicateurs (Bispo et al., 2009).

Parmi les études de bioindication, deux types peuvent être distingués :

- La bioindication passive : elle utilise des organismes polluo-sensibles autochtones qui pourront alerter lors d'un événement de pollution. La surveillance de ces indicateurs permet de révéler une pollution parfois non identifiée lors d'étude d'ERE basée sur les concentrations sans effets observées.
- La bioindication active : elle correspond à l'introduction (encagement *in situ*) d'organismes au passé biologique connu (espèce, âge,…) dans un environnement en conditions standardisées. Les organismes sont exposés durant un temps déterminé. Suite à cette exposition « forcée », les bioindicateurs sont retirés de cet environnement pour étudier les conséquences de cette exposition.

A l'heure actuelle, il existe un besoin de définir des batteries d'indicateurs pour identifier et quantifier les perturbations du sol et leurs impacts sur les écosystèmes. Le sol étant une ressource essentielle pour les sociétés humaines et les écosystèmes, les secteurs économiques et les acteurs publics ont besoin d'outils de caractérisation et d'évaluation des risques environnementaux basés sur les propriétés biologiques du sol. Des travaux sont en cours pour répondre à ces attentes (http://www2.ademe.fr/servlet/KBaseShow?sort=-1&cid=96&m=3&catid=10143). En effet, les indicateurs classiquement utilisés reposent essentiellement sur des propriétés physiques et chimiques du sol alors que les paramètres biologiques intègrent l'ensemble des stress environnementaux (Commission of the European communities, 2006; Bispo et al., 2009). C'est pour répondre à cette demande que le programme ADEME « Bioindicateurs de la qualité des sols » a vu le jour (http://ecobiosoil.univ-rennes1.fr/ADEME-Bioindicateur/). Suite à une première phase de ce programme (Bio 1, 2004-2008) ayant pour but de développer et de tester des batteries d'indicateurs, l'objectif de la seconde phase (Bio2) est de mettre en œuvre sur des sites ateliers communs (sites agricole, forestier et industriel) les outils biologiques sélectionnés lors de la première phase et de valider leur utilisation en fonction de la problématique étudiée (surveillance, caractérisation biologique, étude de risques…). Parmi les problématiques étudiées, le programme Bio2 s'intéresse notamment aux contaminations des sols par les ETM (Gis Sol, 2011) et plus particulièrement à ceux étudiés dans le programme RMQS (Réseau de Mesures de la Qualité des Sols, (Villanneau et al., 2008). Cette seconde phase intègre de nombreux bioindicateurs passifs (communautés microbiennes, végétaux, divers invertébrés dont collemboles, nématodes, vers de terre, micromammifères) ainsi qu'un bioindicateur utilisé en bioindication active : l'escargot *Cantareus aspersus*.

Approche substance et approche matrice et contamination des sols alimentent actuellement l'évaluation des risques environnementaux (ERE) via l'interprétation de l'état des milieux (IEM). Cependant, cette approche de l'ERE n'intègre pas les mécanismes modulant les transferts de contaminant (propriété physico-chimiques des sols, biologie de l'organisme…). Pour prendre en compte ces mécanismes et pour répondre aux besoins des politiques publiques, il est nécessaire de disposer d'outils d'aide à la décision et à la gestion des sites et sols pollués basés sur des mesures biologiques intégratives (dans l'espace et dans le temps) des variables environnementales modulant le transfert des métaux aux organismes. En effet, aucun outil n'a été développé à ce jour pour évaluer le risque d'un transfert de contaminant *in situ* aux invertébrés du sol à un niveau spécifique puisqu'aucune valeur de référence dans les tissus d'organismes sentinelles n'a été déterminée.

IV. L'escargot *Cantareus aspersus* : bioindicateur de la qualité des sols

Parmi les bioindicateurs inféodés au sol déjà utilisés pour l'étude de la qualité des sols (Jeffery et al., 2010), pour appréhender la mobilité des ETM vers les organismes (Peijnenburg et al., 1999c; Vijver et al., 2006; Heink and Kowarik, 2010) ou pour réaliser des études d'ERE (Suárez-Serrano et al., 2009; Coeurdassier et al., 2010), notre laboratoire s'est focalisé sur le développement d'un bioindicateur présent à l'interface sol-plante-atmosphère, l'escargot petit gris (*Cantareus aspersus* = *Helix aspersa*). Les escargots sont des mollusques gastéropodes pulmonés qui répondent aux critères définis par Hopkin (Hopkin, 1993) d'un bon bioindicateur (Gomot-de Vaufleury, 2000):

- participer activement au fonctionnement de l'écosystème,
- être sédentaire, largement distribué et facile à identifier et à échantillonner,
- être bioaccumulateur,
- être tolérant aux fortes contaminations de l'environnement,
- leur écologie et physiologie doivent être connues.

Les escargots ont donc depuis longtemps été utilisés pour étudier l'accumulation de polluants (Coughtrey and Martin, 1976; Dallinger et al., 1993; Laskowski and Hopkin, 1996; Coeurdassier et al., 2002; de Vaufleury et al., 2006; Gimbert, 2006; Druart et al., 2011).

Les escargots utilisés au cours de cette thèse sont des mollusques gastéropodes pulmonés terrestres et appartiennent à l'espèce *Cantareus aspersus* (*syn. Helix aspersa*), plus communément appelé escargot de jardin ou escargot Petit gris. C'est une espèce ubiquiste qui s'adapte à des milieux, des sols et des climats variés et s'accommode très bien de milieux fortement anthropisés, mais semble cependant préférer les milieux ouverts. L'escargot est très répandu en Europe, en particulier dans les régions méditerranéennes et océaniques. Il peut être considéré comme une peste dans les jardins. On le retrouve également dans les dunes, les bois, les rochers, les haies mais également dans les zones cultivées (Kerney et al., 2006). Leur coquille, dextre, est de couleur brun-jaunâtre avec un diamètre variant de 20 à 35 mm et une hauteur de 25 à 40 mm. Deux grandes parties sont distinguées : le pied et les viscères (Figure 4). Les viscères correspondent aux organes situés à l'intérieur de la coquille et comprennent le rein, l'hépatopancréas (site prinicipal de stockage des métaux), le coeur et une partie de l'appareil génital qui se prolonge également dans le pied. Le pied comporte essentiellement la sole pédieuse, musculeuse, la partie antérieure du tube digestif et le système nerveux.

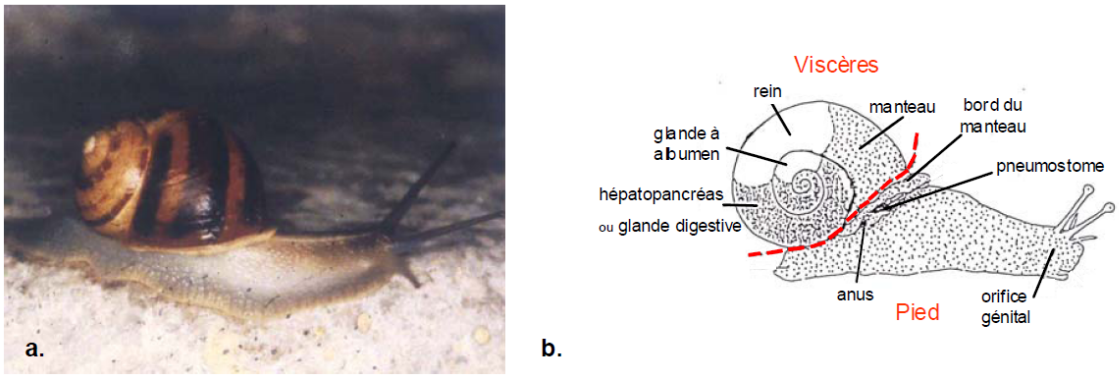


Figure 4. a. *Cantareus aspersus* adulte. B. Schéma d'un individu sorti de sa coquille, le tireté rouge indique la marque de dissection pour la séparation pied/viscères.

Leur activité est grandement conditionnée par les conditions environnementales telles que l'hygrométrie du sol et de l'air (activité au dessus de 80% d'humidité), la température (inactivité pour des températures nocturnes inférieures à 9°C) et l'intensité lumineuse (Chevallier, 1982). L'estivation et l'hibernation de cette espèce sont provoquées par des températures supérieures à 15°C (accompagnées d'une absence prolongée d'humidité) ou inférieures à 5°C (et la diminution de la photopériode) entrainant un fonctionnement minimal du métabolisme. En estivation, les escargots se fixent sur un support sec et secrètent un épiphragme (voile de mucus solidifié) ; en hibernation, l'épiphragme est plus solide et les individus s'enfouissent dans le sol ou la litière.

La croissance des escargots est caractérisée par 4 phases. Ils sont appelés « jeunes éclos » jusqu'à environ 1 g (correspondant à 1 mois), puis sont qualifiés de juvéniles jusqu'à environ 5 g et de subadultes jusqu'à 8-9 g. Lorsque les escargots atteignent la taille adulte après environ 4 mois de croissance en conditions contrôlées favorables, ils se bordent (phénomène caractérisé par le bord de la coquille qui s'incurve et par un épaississement du bord du manteau). La croissance de la coquille s'arrête alors, du moins en longueur, et les escargots, devenus adultes, terminent leur maturation sexuelle et deviennent aptes à se reproduire.

En milieu naturel, la croissance est beaucoup plus longue et dure de 1 à 2 ans selon les conditions climatiques. La durée de vie en milieu naturel est en moyenne de 6 à 7 ans. La reproduction de *C. aspersus* en milieu naturel a lieu de mai à mi-septembre. Chez ces hermaphrodites protandres la fécondation est réciproque par échange de spermatophores entre les 2 partenaires, certains individus pouvant toutefois se comporter uniquement comme mâle ou comme femelle. La durée entre l'accouplement et la ponte est d'environ 10 jours en conditions optimales mais peut atteindre

1 voire 2 mois lorsque les conditions de l'environnement sont défavorables. La ponte peut durer jusqu'à 36 heures. Les œufs (entre 80 et 130) sont déposés dans une cavité creusée à quelques cm sous la surface du sol, cavité qui est ensuite rebouchée. L'incubation dure de 12 jours à 20°C à environ 22 jours à 15°C. Le nombre moyen de ponte par reproducteur est de 1,3 et le nombre d'infantiles produits est de 75 à 85 (Madec, 1983).

Son régime alimentaire n'est pas spécialisé et l'escargot s'adapte en fonction des plantes qui colonisent le milieu (Chevalier et al., 2001). Le sol fait également partie de son alimentation et peut influencer sa croissance (Gomot et al., 1989). Des sols pauvres en calcium peuvent constituer un facteur limitant à la croissance des escargots, le calcium étant un élément indispensable à la formation de la coquille. Ce régime alimentaire basé sur la consommation de plante et de sol en fait un bioindicateur très intéressant car ils intègrent plusieurs sources de contaminations accessibles *via* différentes voies d'exposition (Figure 5) :

- digestive (voie principale, >80%) par ingestion de nourriture (plantes (Scheifler et al., 2006)) mais également particules de sols (Gomot et al., 1989)),
- cutanée (20% maximum) par diffusion des polluants du sol à travers l'épithélium du pied (Coeurdassier et al., 2002; Gomot-de Vaufleury and Pihan, 2002),
- respiratoire par inhalation de gaz et/ou de particules atmosphériques (Ismert et al., 2002; Regoli et al., 2006).

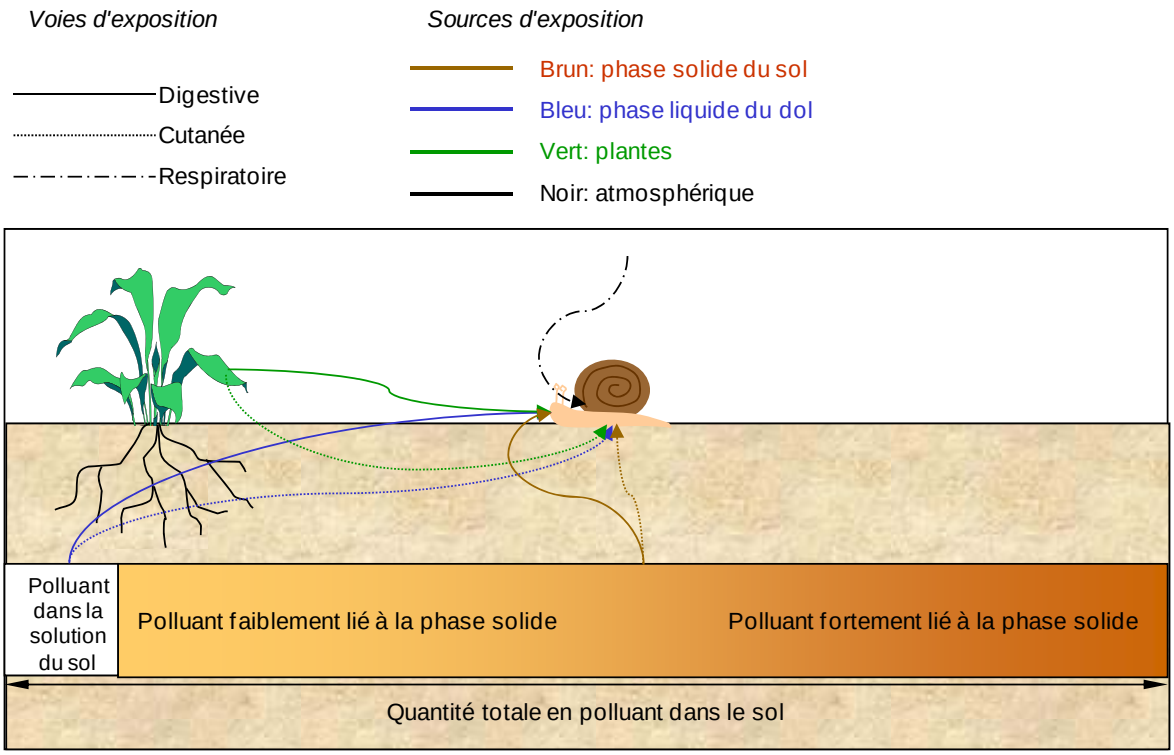


Figure 5 : Voies et sources de transfert des polluants aux escargots (Scheifler, 2002).

Les escargots sont ont de fortes capacités de bioaccumulation pour de nombreux polluants (Gomot and Pihan, 1997; Coeurdassier et al., 2002; Viard et al., 2004), en effet, il a été observé qu'environ 68% du Cd, 90% du Cu, 43% du Pb et 60% du Zn ingérés sont accumulés (Laskowski and Hopkin, 1996) ce qui les classe parmi les macroconcentrateurs pour plusieurs métaux comme le Cd ou le Cu (Dallinger, 1993). Le fait d'une bonne résistance à l'apparition d'effet lié à l'accumulation de métaux par les escargots (CE 50 : 140 mg $Cd.kg^{-1}$, 355 mg $Cr.kg^{-1}$, 1200 mg $Cu.kg^{-1}$, >30000 mg $Pb.kg^{-1}$ et 5500 mg $Zn.kg^{-1}$ dans la nourriture), peut engendrer un empoisonnement de leur prédateurs (Laskowski

and Hopkin, 1996). En effet, l'escargot compte de nombreux prédateurs, aussi bien des vertébrés, comme les oiseaux (grive), les petits mammifères (musaraigne, hérisson), les reptiles (lézard, couleuvre), que des invertébrés, comme les carabes, les vers luisants et les limaces (Chevallier, 1992; Barker, 2004). Staikou et Lazaridou-Dimitriadou (Staikou and Lazaridou-Dimitriadou, 1989) soulignent leur rôle dans les transferts de matière et d'énergie depuis les producteurs vers les niveaux trophiques supérieurs. Ceci qui suggère une implication potentielle des gastéropodes dans le transfert des polluants métalliques le long des chaînes trophiques (Beeby, 1985; Laskowski and Hopkin, 1996). L'homme consomme également l'escargot Petit Gris dont lachair est très appréciée, bien qu'en quantité moindre (15 tonnes de conserves en France en 2008) que l'escargot de Bourgogne *Helix pomatia* (876 tonnes de conserves en France en 2008, Druart et al., 2011).

L'utilisation de l'escargot *C. aspersus* comme bioindicateur peut apporter des éléments pour améliorer l'ERE, par exemple sur la biodisponibilité d'un polluant dans un environnement pour cet organisme. En effet, pour qu'il y ait un transfert de polluant, il faut que plusieurs paramètres soient réunis : une contamination, une exposition et une assimilation. C'est à ce niveau que le concept de biodisponibilité prend tout son intérêt car sa mesure permet de caractériser la capacité d'un organisme à assimiler un contaminant. On peut alors observer et modéliser des accumulations de contaminant dans l'organisme, définir les effets de cette accumulation et ainsi effectuer une évaluation des risques complète.

Parmi le panel des bioindicateurs de la qualité des sols, l'escargot se situe à l'interface sol-plantes-air. Même si de nombreux travaux ont étudié l'accumulation et le devenir des métaux chez l'escargot, de nombreuses inconnues persistent :
- l'influence des paramètres des sols sur la contribution des différentes sources de contamination et sur le transfert de métaux du sol aux escargots n'a jamais été étudiée alors que ce sont ces paramètres qui modulent la mobilité des métaux
- l'assimilation et l'excrétion *in situ* de certains métaux comme l'As, le Cr ou le Sb n'ont jamais été étudiées à notre connaissance.
De plus, il n'existe pas à ce jour de valeurs de concentrations internes de référence en métaux pour les escargots exposés en bioindication active. Ces valeurs seraient pourtant très utiles pour mettre en évidence la biodisponibilité des métaux sur divers sites.

V. Le concept de biodisponibilité

La biodisponibilité est un concept permettant de caractériser l'exposition d'un organisme à un contaminant et donc un risque de transfert de contaminants d'un compartiment de la biosphère aux organismes. Elle permet d'intégrer les concentrations de polluants, leurs flux et leurs devenirs dans un écosystème grâce à des mesures biologiques et chimiques.

V.1. Définition du concept

Une des raisons à l'origine de l'intérêt porté à la biodisponibilité est le constat que seule une fraction de la contamination totale est transférable (et donc potentiellement toxique) aux organismes. Cette fraction a été définie comme étant la fraction biodisponible d'un polluant (ou pool disponible) (ISO 17402, 2008). L'étude de la biodisponibilité permet une description plus exacte et plus réaliste de l'exposition d'un organisme à un environnement contaminé (de Vaufleury et al., 2009; Pauget et al., 2011).
En 2003, Peijnenburg et Jager (Peijnenburg and Jager, 2003b) définissent le pool biodisponible comme étant la part de la quantité totale d'un ETM présent dans un compartiment environnemental spécifique qui, dans un laps de temps donné, est disponible (ou rendue disponible) pour être

assimilée par un organisme à partir de son environnement immédiat (exposition cutanée) et/ou par ingestion de nourriture (voie digestive). La biodisponibilité décrit les processus complexes d'assimilation, de séquestration et d'excrétion des ETM dans un organisme qui sont modulés par les propriétés des ETM considérés (métal essentiel ou non, spéciation…), les propriétés physico-chimiques du sol (pH, CEC…), le temps d'exposition et la physiologie de l'organisme considéré. La fraction biodisponible (ou pool disponible) d'un métal d'un sol représente la fraction de ce métal qui est potentiellement disponible pour être assimilée par l'organisme (Frische et al., 2003).
La biodisponibilité se décompose en trois volets intégratifs de ces processus. Ces trois volets sont la disponibilité environnementale, la biodisponibilité environnementale et la biodisponibilité toxicologique (Figure 6).

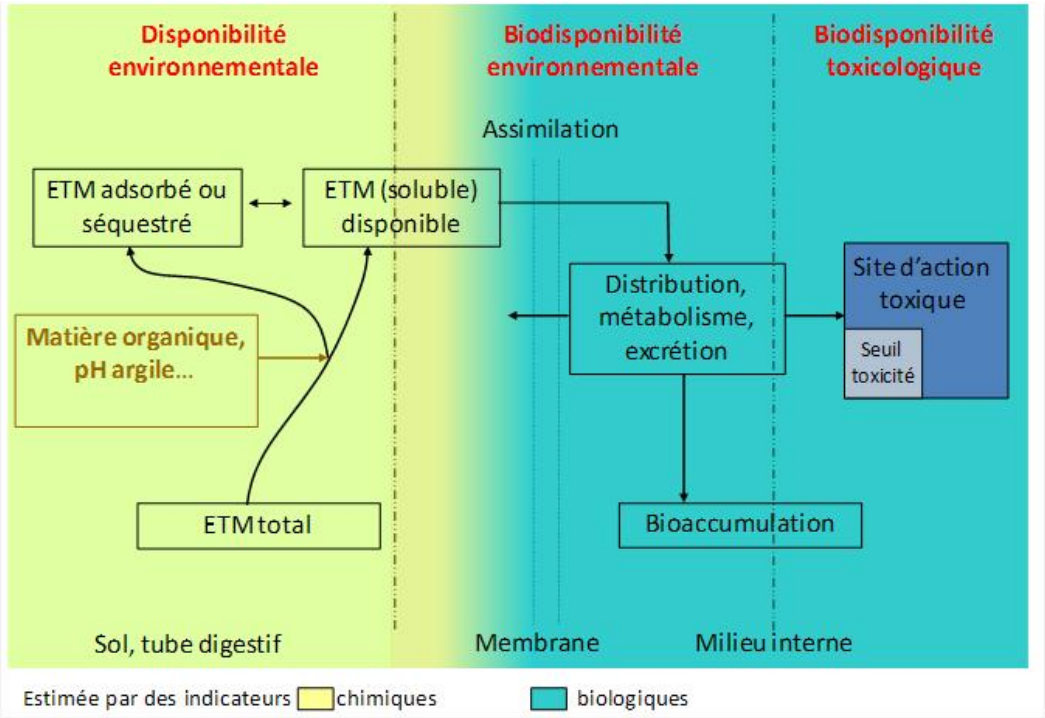


Figure 6 : Schéma des trois volets de la biodisponibilité. Adapté de Lanno et al. (Lanno et al., 2004).

V.2. La disponibilité environnementale des ETM

V.2.a. Définition

La disponibilité environnementale décrit l'ensemble des processus physico-chimiques qui conditionnent la répartition du polluant entre les différentes phases du sol (phases solide et liquide). Elle se réfère à la mobilité du polluant présent dans le sol et à sa capacité d'interaction avec les autres phases du sol ainsi qu'à la spéciation du métal. La disponibilité environnementale représente le pool disponible de l'ETM considéré qui est potentiellement transférable à un organisme dans un temps et dans des conditions données. Ce pool est spécifique à chaque sol (selon ses caractéristiques édaphiques), ainsi qu'à chaque ETM (selon ses caractéristiques intrinsèques) et possède une dynamique variable changeante avec les conditions environnementales (Fairbrother et al., 2007; Smith, 2009).
L'escargot vivant à l'interface sol-plante-air, toutes les sources de contamination vont donc moduler la quantité d'ETM totale disponible pour cet organisme.

V.2.b. Facteurs de variations

Le principal facteur qui influence la disponibilité environnementale des ETM du sol est leur spéciation. Elle va grandement conditionner la localisation et la mobilité des ETM dans les sols.

- *Spéciation des ETM dans les sols*

La spéciation définit l'état de valence d'un élément ou son association avec les constituant du sol (Denys et al., 2009b; Caboche et al., 2010). La répartition des ETM entre les différents constituants d'un sol va dépendre de leurs caractéristiques intrinsèques (masse, charge, rayon atomique). Ainsi chaque ETM va avoir des affinités particulières avec les différents constituants du sol (Tableau 2). Ces affinités vont définir les sites de fixation des ETM au sol et implicitement leur mobilité. La spéciation des ETM est un paramètre important à prendre en compte car les ETM ne présentent pas la même mobilité et toxicité selon leur forme.

La spéciation des ETM est modulée par les paramètres édaphiques (Sterckeman et al., 2004; Linde et al., 2007). Ainsi, le pH, la capacité d'échange cationique (CEC), et les conditions redox vont influencer la forme chimique des métaux (Girard et al., 2005 ; Giacomino et al., 2010) et leur mobilité dans les sols et incidemment leur transfert aux organismes (Bur, 2008; Pareuil et al., 2008). Par exemple, la forme la plus toxique de l'As (l'As III,(INERIS, 2010)) va être trouvée dans les sols présentant des potentiels réducteurs alors que des sols à potentiel oxydant et à pH alcalin vont contenir de l'As sous sa forme pentavalente (As V), forme peu toxique et moins mobile (INERIS, 2006, 2010).

Tableau 2 : Formes chimiques dans le sol de différents ETM et principaux compartiments de sol associés. Adapté de (Bur, 2008)

Elément	Forme chimique dans le sol	Forme chimique dans la solution du sol	Principales phases porteuses
Antimoine	Sb_2O_5, Sb_2O_3 Sb_2S_5, Sb_2S_3	complexe avec les acides humiques	MO, argiles, hydroxydes
Arsenic	As V si milieu oxydant ($H_2As0_4^-$, $HAsO_4^{2-}$), As III si milieu réducteur ($HAsO_2$, AsO_2^-)	Toutes formes selon les caractéristiques physico-chimiques du milieu (pH, Eh…)	MO, argiles, hydroxydes
Cadmium	Cd^{2+}, $CdSO_4^{2-}$, $CdCl^+$; $CdHCO_3^+$, CdO, $CdCO_3$, $Cd(PO_4)_2$, CdS, $CdCl_2$	Cd^{2+} et/ou chélates d'acides fulviques	MO, argiles, carbonates
Chrome	Principalement CrIII et un peu de CrVI	CrVI majoritairement	MO, argiles
Cuivre	Cu^{2+}, $CuSO_4$, $CuCO_3$, $Cu(OH)_2$	Chélates de composés organiques solubles ou Cu^{2+}	Oxydes Fe et Al, MO, argiles
Plomb	Pb^{2+}, $PbHCO_3^+$, $PbOH^+$, PbS, $PbSO_4$, $Pb(OH)_2$, $PbCO_3$, PbO, $Pb(PO_4)_2$, $PbCl^+$	Pb^{2+} et/ou chélates d'acides fulviques	Oxydes Fe et Al, MO, argiles
Zinc	Zn^{2+}, $ZnSO_4$, $ZnHCO_3^+$, $ZnCO_3$, $ZnFe_2O_4$, $ZnSIO_4$, $Zn_3(PO_4)_2$	Chélates de composés organiques solubles ou Zn^{2+}	Oxydes de Fe, MO, argiles

- *Localisation et mobilité*

Dans les sols, les ETM se répartissent entre la phase solide et la phase liquide. La plupart du temps, la quantité de métaux présente dans la solution du sol ne représente qu'une faible partie de la quantité totale présente dans le sol (Morel, 1998; Girard et al., 2005). La majeure partie des ETM va donc se concentrer dans la phase solide du sol et se répartir entre ses différents constituants (Figure 7). Les principaux acteurs de la fixation et de la rétention des métaux dans un sol sont :

Les constituants de la phase solide

- Les minéraux primaires : les ETM du sol inclus dans les minéraux primaires sont très peu mobiles et disponibles. Cependant, l'érosion et la dégradation de ces minéraux engendrent un relargage des métaux dans le sol.
- Le complexe argilo-humique : c'est une association de minéraux argileux et de macro molécules organiques possédant de fortes capacités de rétention des ETM. Ses propriétés chimiques de surface particulières permettent le piégeage des ETM sous forme échangeable (McBride, 1995). Les différents types d'argile et de matière organique (MO) qui composent ce complexe font varier la CEC modulant la mobilité et les migrations d'ETM au sein du sol. En effet, MO et argile offrent de nombreux sites de liaison pour les métaux grâce à leurs charges négatives. Ainsi les métaux sous formes cationiques (Cd^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+}…) vont se fixer de manière réversible sur la MO et les argiles (Bradl, 2004). Cependant, cette fixation des métaux est dépendante du type de MO et d'argile. Il a par exemple été démontré que parmi les argiles, la montmorillonite fixe plus efficacement les métaux que la kaolinite (Bhattacharyya and Gupta, 2008; Giacomino et al., 2010). Quant à la MO des sols, elle a des conséquences variées sur la mobilité, la solubilité ainsi que la biodisponibilité des métaux (Impellitteri et al., 2002). Il a été démontré que la taille de la MO a une nette influence sur sa capacité à fixer les métaux (Quenea et al., 2009). Ainsi, plus la taille de la MO est faible, plus la force de fixation et donc de rétention des métaux sera forte (Labanowski et al., 2007). Cependant, la MO présente sous forme soluble, de par ses propriétés de complexation, peut favoriser la mobilité des métaux en augmentant leur concentration dans la solution du sol (Girard et al., 2005).
- Les oxydes : les oxydes (fer, aluminium,…) sont des solides ioniques dont la charge dépend de la nature du minéral et des conditions physico-chimiques dans lesquels ils se trouvent. Ce sont des sites de fixation des ETM par précipitation d'un complexe oxyde-ETM.

La phase liquide ou solution du sol

Même si elle ne contient généralement qu'une faible proportion des ETM totaux, c'est dans cette phase aqueuse que les ETM sont les plus mobiles et donc généralement les plus disponibles pour les organismes (van Straalen and van Gestel, 1998). C'est ici que sont mis en jeu la plupart des processus pédologiques qui vont conditionner la répartition des ETM entre les différents compartiments du sol. En solution, les ETM sont présents sous formes de cations, d'hydroxydes ou d'oxyanions selon leurs propriétés physiques et chimiques (Pedro and Delmas, 1970; Fairbrother et al., 2007) ou sous forme colloïdale associée à de la MO (Girard et al., 2005).

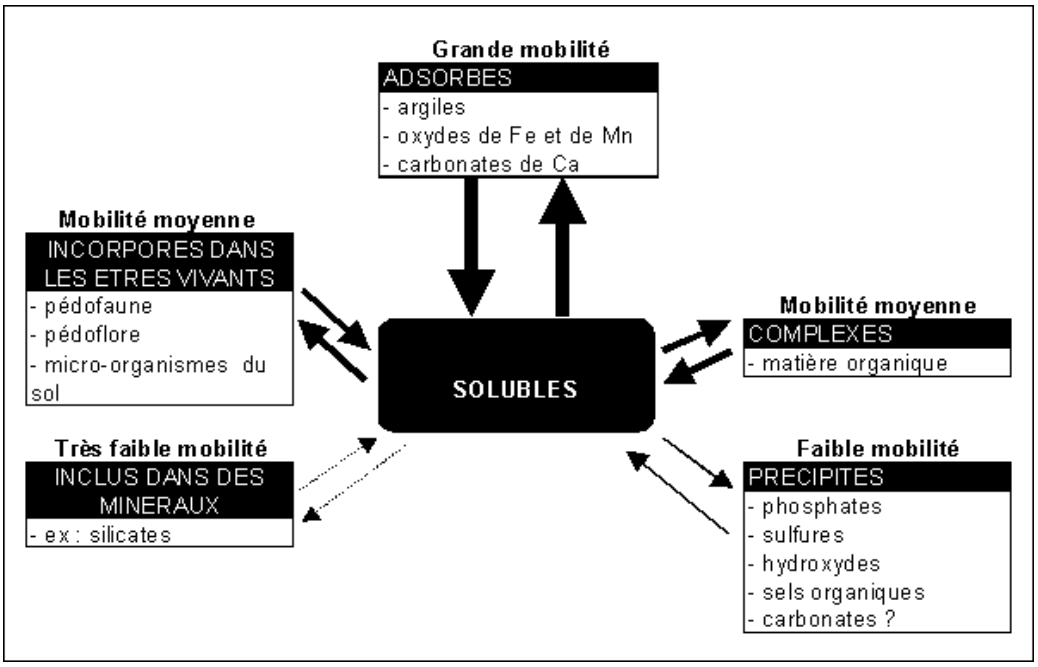


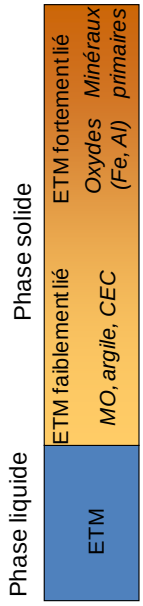
Figure 7 : Répartition et mobilité des ETM entre les différentes fractions d'un sol (Juste, 1995).

V.2.c. Mesure de la disponibilité environnementale

La mesure de la disponibilité environnementale par des extractants chimiques de différentes forces ou par des techniques de dilution isotopique (Tongtavee et al., 2005) permet d'estimer la répartition ainsi que la mobilité des métaux dans les sols (Douay et al., 2009). Même si la concentration totale en ETM dans les sols est encore utilisée majoritairement lors de l'ERE, de nombreux auteurs préconisent l'estimation du pool disponible (fraction d'un contaminant dans le sol potentiellement transférable aux organismes) lors d'étude de biodisponibilité (Frische et al., 2003; Janssen et al., 2003; Lanno et al., 2004; Pueyo et al., 2004; Gupta and Sinha, 2007; Berthelot et al., 2008). Cependant, un des objectifs de la mesure de la disponibilité environnementale est de savoir si une mesure chimique permettrait de s'affranchir de mesures biologiques pour déterminer la biodisponibilité des métaux pour les organismes dans l'ERE. A terme et dans l'affirmative, les mesures chimiques permettant de prédire la biodisponibilité des métaux pour les organismes seraient privilégiées car plus simples et moins couteuses à réaliser.

Ainsi on tente d'estimer le pool d'ETM biodisponible pour « un organisme » par des extractions des ETM du sol avec des sels neutres comme le $CaCl_2$ 0,01 M (Houba et al., 2000; Degryse et al., 2009), le $NaNO_3$ 0,1 M ou le NH_4NO_3 1 M (Lebourg et al., 1998; Pueyo et al., 2004) ; des acides dilués comme le HCl ou le HNO_3 (Tipping et al., 2003), des extractants organiques comme le DTPA ou l'EDTA (Manouchehri et al., 2006; Santos et al., 2010) ou par des extractions séquentielles utilisant plusieurs extractants dans le but d'identifier les principales phases du sol porteuses de métaux (Scheifler, 2002). Ces différentes méthodes d'extraction d'ETM dans les sols tentent de prédire la biodisponibilité des ETM tant chez les végétaux que chez les animaux et certains pays d'Europe ont normalisé leurs utilisations (Tableau 3) (VSBo (Verordnung über Schadstoffgehalt im Boden), 1986; NEN 5704, 1996; DIN 19730 (Deutsches Institüt für Normung), 1997; Quevauviller et al., 1998).

Tableau 3 : Méthodes chimique utilisées pour mesurer la disponibilité environnementale et prédire la biodisponibilité des métaux pour des organismes.



Méthode	Type d'extractant	Application
concentration totale	acide	actuellement utilisée en évaluation des risques
extraction à l'EDTA	organique	biodisponibilité pour les plantes (Quevauviller et al., 1998)
extraction au NH_4NO_3	sel neutre	normalisée en Allemagne pour le transfert aux végétaux (DIN 19730, 1997)
extraction au $CaCl_2$	sel neutre	normalisée aux Pays-Bas pour estimer la fraction biodisponible (NEN 5704, 1996)
extraction au $NaNO_3$	sel neutre	normalisée en Suisse pour estimer les risques phytotoxiques (VSBo, 1986)
activité ionique dans la solution du sol	estimation	simulation du pool labile (cations bivalents) (Ge et al., 2000)
concentration totale dissoute dans la solution du sol	estimation	simulation du pool labile (ETM dissous dans la solution du sol) (Sauvé et al., 2000)

L'avantage de toutes ces méthodes est la possibilité d'estimer les différentes fractions du sol qui entreraient en jeu dans l'étude de la biodisponibilité. Grâce aux extractants chimiques, on peut tenter de simuler le pool disponible du sol (Scheifler, 2002) par l'estimation des métaux présents dans ses différents constituants (Figure 7 et Tableau 3). Cependant, il a été observé que si un extractant chimique pouvait prédire la biodisponibilité des métaux pour un organisme dans une gamme de contamination de sol (Figure 8, zone B), il pouvait perdre sa capacité de prédiction dans des gammes de contaminations plus fortes (Figure 8, zone C). D'autres techniques ont également été développées afin d'estimer la biodisponibilité via les processus d'assimilation de métaux comme le « Free Ion Activity Model » (FIAM, (Lofts et al., 2004) mais qui ne considérait que les formes métalliques en solution. Beaucoup d'études ont été réalisées selon la théorie de la partition à l'équilibre (equilibrium partition theory, EPT,(Di Toro et al., 1991)) partant du principe que le polluant est en équilibre cinétique entre la phase solide et liquide du sol et que l'organisme est exposé très majoritairement au métal via la phase liquide du sol (Pore water hypothesis, PWH,(Frische et al., 2003)). Grâce à ce modèle, la biodisponibilité d'un polluant pourrait être prédite par le coefficient de partition (Kp : rapport de la concentration du métal dans la phase liquide sur la concentration de métal sur la phase solide). Cependant, même si des résultats prometteurs ont été obtenus au départ, il s'est avéré que ce modèle n'est pas utilisable pour tous les métaux ni pour tous les organismes (Crommentuijn et al., 1997; Janssen et al., 1997a; Peijnenburg et al., 1999b; Oste et al., 2001). Des techniques biomimétiques sont également en cours d'étude afin de pouvoir estimer la biodisponibilité des ETM en simulant leur transfert à travers une membrane biologique. C'est par exemple le cas de la technique par gradient de diffusion sur couche mince (Diffusive gradient in thin films (DGT)) (Roulier et al., 2008; Duquène et al., 2010; Roulier et al., 2010; Bade et al., 2012). De même, le test *in vitro* BARGE unifié (UBM) simule la physiologie du tractus gastro-intestinal de l'enfant afin d'estimer la bioaccessibilité des métaux pour un enfant de 0-6 ans (Caboche, 2009).

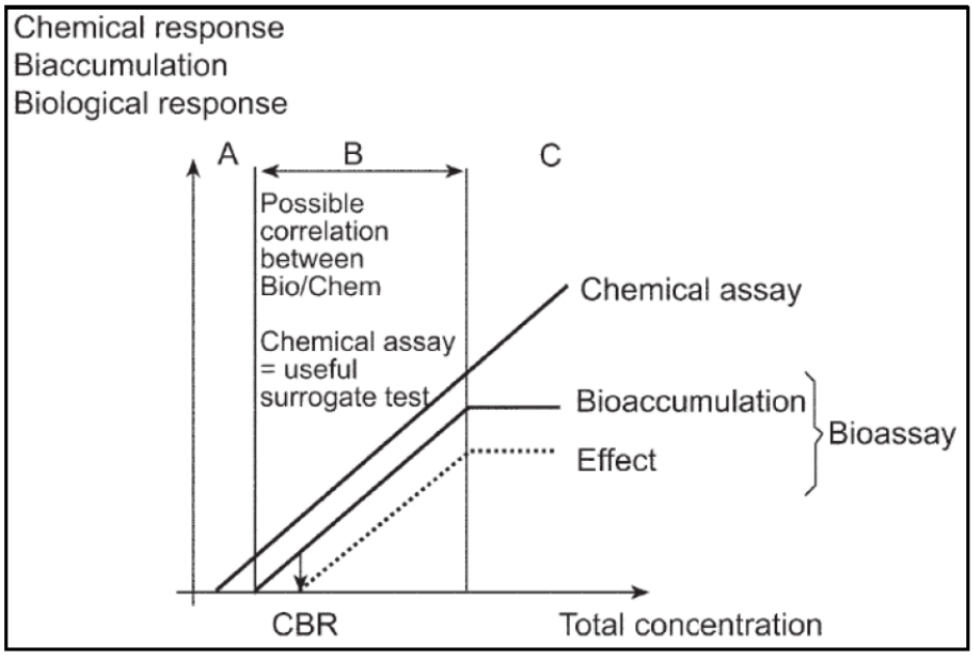


Figure 8 : Méthodes d'évaluation de la biodisponibilité : relations conceptuelles entre tests chimiques, biologiques et bioaccumulation (Harmsen, 2007). Réponses des tests chimiques et biologiques en fonction de la concentration totale. Zone A : le contaminant est détecté, mais il n'y a pas d'effet détectable. Zone B : les courbes dose-réponse sont parallèles pour les tests chimiques et biologiques, alors le test chimique constitue un substitut utile aux essais biologiques, il est représentatif des réponses biologiques (accumulation ou effets). Zone C : les relations entre les tests deviennent non linéaires, la corrélation entre les paramètres d'évaluation de la biodisponibilité est perdue. CBR : critical body residues (concentration interne à partir de laquelle un effet est observé).

Cependant, même si de nombreuses méthodes sont utilisées en routine pour estimer la biodisponibilité des métaux pour différents organismes (Tableau 3), il n'existe actuellement pas de méthode consensuelle pour estimer la biodisponibilité des métaux (van Gestel, 2008). Par exemple, lors d'une étude préliminaire en laboratoire (Pauget et al., 2011) et une seconde *in situ* (Mourier et al., 2011), il a été démontré qu'une extraction au $CaCl_2$, pourtant recommandée par l'ISO pour estimer la fraction de métal du sol biodisponible pour certains organismes et certains métaux (ISO 17402, 2008), n'était pas représentative de la réelle biodisponibilité du Cd et Pb pour l'escargot.

Dans l'optique d'évaluer les risques liés aux métaux, les politiques publiques se tournent vers des méthodes chimiques d'estimation de la biodisponibilité. Cependant, il n'existe pas à ce jour de méthodes permettant d'évaluer la biodisponibilité des métaux pour les organismes en général. Il existe donc un besoin d'étudier et de caractériser la capacité des extractants à estimer la biodisponibilité des métaux pour l'escargot afin de valider des couples extractant-métal. Dans le but d'utiliser des mesures chimiques pour simuler la biodisponibilité des métaux, il apparait nécessaire de caractériser les influences de chaque variable (caractéristiques des sols, spéciation des contaminants…) sur le pool disponible. L'influence des paramètres des sols sur la disponibilité des métaux dans les sols et le lien avec la biodisponibilité pour l'escargot n'a été étudiée qu'une seule fois en laboratoire (Pauget et al., 2011) et qu'une seule fois *in situ* avec des individus autochtones de différentes espèces (Mourier et al., 2011) et pour seulement trois métaux (Cd, Pb et Zn). Il existe donc un réel besoin de caractériser les paramètres des sols qui modulent la biodisponibilité des métaux.

V.3. La biodisponibilité environnementale des ETM

V.3.a. Définition

La biodisponibilité environnementale correspond aux processus physiologiques propres à l'organisme considéré qui vont conditionner les phénomènes d'absorption et d'assimilation et d'excrétion. Dans cette thèse, une distinction sera faite entre absorption et assimilation : l'absorption correspond principalement à la quantité de nourriture (sol et végétaux) ingérée (*i.e.* ingestion, reflète l'exposition par voie digestive) et l'assimilation qui correspond à l'ensemble des ETM se retrouvant dans le milieu interne de l'escargot (*i.e.* passage des membranes biologiques, intègre l'ensemble des voies de contamination). Quand à l'excrétion, elle correspond à l'évacuation du métal de l'organisme. Pour estimer cette biodisponibilité environnementale, des mesures des concentrations internes en ETM sont réalisées après une exposition déterminée ou plusieurs fois au fil de l'exposition en fonction de l'étude de biodisponibilité souhaitée. Ces deux méthodes différentes seront détaillées en section V.3.c. Cette mesure est donc intégrative de la disponibilité environnementale et des paramètres qui la module puisque l'assimilation des métaux dépend de leur accessibilité dans les sols et de leur répartition entre les différentes sources de contamination.

V.3.b. Facteurs de variations

La biodisponibilité environnementale va être conditionnée en majeure partie par le récepteur écologique concerné ainsi que la nature des ETM et éventuellement les interactions entre ETM. En effet, c'est la capacité d'un organisme à prélever les métaux dans les sols puis à les assimiler qui module la biodisponibilité environnementale. De même, le mode de vie des organismes entre en jeu. Pour une espèce inféodée au sol, l'exposition sera plus importante que pour une espèce peu en contact avec le sol, de par le temps d'exposition mais également par les différentes sources de contamination (sol, humus, végétaux…) (Peakall and Burger, 2003; Smith et al., 2007). En effet, il existe des différences de voies d'exposition entre les organismes. Par exemple, chez les vers de terres la voie principale de contamination est la voie cutanée (Vijver et al., 2003) tandis que pour l'escargot c'est la voie digestive qui prédomine (Coeurdassier et al., 2002; Gomot-de Vaufleury and Pihan, 2002). De plus, il a été démontré chez l'escargot des variations dans la contribution des sources de contamination en fonction du métal étudié (Scheifler et al., 2006).

- *Sources de contamination*

La source de contamination est très importante à prendre en compte car elle va grandement déterminer l'accumulation des ETM chez les organismes. *In situ*, les escargots sont exposés au sol mais également aux contaminants provenant des végétaux qu'ils ingèrent (Figure 5). La concentration en ETM des végétaux ainsi que la composition du cortège floristique ingéré modifie la quantité de métal disponible pour l'escargot. En effet, si les végétaux du site ne sont pas des espèces accumulatrices, l'exposition par la végétation va être faible et inversement. Il a également été démontré que les escargots vont consommer des poacées (Chevalier et al., 2001) ainsi que d'autres espèces comme *Urtica dioca* et *Thlaspi caerulescens* (Noret et al., 2005; Sinnett et al., 2009) qui peuvent accumuler de grandes quantités de métaux. La concentration et la forme de stockage dans les tissus de la plante consommée vont donc être des facteurs de variation de l'exposition des escargots. De plus, chez *C. aspersus*, il a été démontré des variations dans les contribution des source de contamination en fonction des métaux : en conditions contrôlées qu'environ 80% du Pb accumulé proviendrait du sol tandis qu'au moins 60% du Cd accumulé proviendrait de la végétation (Scheifler et al., 2006). Parmi les voies de contamination, c'est la voie digestive qui est responsable de la majeure partie de l'accumulation du Cd (Coeurdassier et al., 2002), cependant la voie cutanée n'est pas négligeable pour autant car il a été démontré que les ETM dans la solution sol sous forme ionique

ou sous forme de complexes organométalliques passent à travers l'épithélium du pied par diffusion passive (Ireland, 1982; Hopkin, 1989) ou par passage facilité par des transporteurs membranaires (Simkiss and Taylor, 1989; Rainbow and Dallinger, 1993).

- *Devenir des ETM après assimilation*

L'évaluation des risques liés aux métaux nécessite de distinguer les ETM essentiels des non essentiels. Parmi les ETM, le chrome (Cr), le cobalt (Co), le cuivre (Cu), le fer (Fe), le manganèse (Mn), le molybdène (Mo), le sélénium (Se) et le zinc (Zn) sont des métaux essentiels nécessaires, à des concentrations physiologiques, au bon développement des organismes alors que d'autres ETM comme l'As, le Cd, le Pb ou le Sb ne sont pas indispensables au fonctionnement des organismes (Mertz, 1981). Après avoir été assimilés, les ETM vont être disponibles pour le métabolisme. Une fois que leur assimilation aura dépassé les besoins métaboliques, ils seront pris en charge de différentes manières par l'organisme en vue d'une détoxication. Nous avons vu précédemment que les ETM pouvaient être classés en trois classes différentes selon leurs affinités pour les différents ligands (section II.1.). Une fois assimilés, les ETM vont circuler dans l'hémolymphe en direction des tissus ou des organes cibles dans lesquels ils seront pris en charge ou exerceront leurs effets toxiques. Dans toutes les études utilisant l'escargot comme indicateur, la glande digestive (*i.e.* l'hépatopancréas) a été reconnue comme étant l'organe principal de stockage des ETM car il contient systématiquement les plus fortes concentrations en Cd, Pb et Zn (Coughtrey and Martin, 1976; Cooke et al., 1979; Gimbert et al., 2008c). C'est dans cet organe que la grande majorité des processus de gestion des ETM vont s'effectuer. Différents mécanismes sont mis en jeu : une séquestration de l'ETM par les métallothionéines ou les granules, une excrétion… Ces différences de séquestration et de prise en charge des ETM sont essentielles à prendre en compte lors d'évaluation des transferts de métaux aux organismes car elles conditionnent, les cinétiques d'accumulation des ETM (section V.3.c., *Evaluation cinétique*). On observe des organismes régulateurs qui maintiennent leurs concentrations en ETM stables même dans des cas de forte exposition et les non régulateurs qui accumulent de grandes quantités de métaux sous forme séquestrés dans des structures spécifiques (Dallinger et al., 1993).

- *Les mécanismes de stockage des ETM*

Plusieurs unités de stockage sont mises en œuvre par les escargots en fonction des métaux (Tableau 4) :

Les granules

Les granules sont des structures sphériques denses de séquestration des métaux présents chez les escargots. Ils ont été classés en quatre catégories par Hopkin (Hopkin, 1989):

- Les granules de type A sont constitués de couches concentriques de phosphates de calcium et de magnésium, et vont séquestrer des métaux de classe A et intermédiaire (Pb, As, Zn,…).
- Les granules de type B contiennent de grandes quantités de soufre en association avec des métaux de classe B et intermédiaire (Cd, As, Pb, Zn,…)..
- Les granules de type C sont composé quasiment exclusivement de Fe. Ils peuvent cependant servir à séquestrer des ETM lors de très forte exposition. Ces granules sont surtout des sites de stockage du Fe et n'ont pas pour fonction première de détoxifier les ETM.

Les métallothionéines (MT)

Ce sont des protéines soufrées capables de fixer des ions métalliques et dont la synthèse est induite généralement par les métaux (Cd, Cu, Zn, Hg... (Dallinger et al., 2004a; Dallinger et al., 2004b)). Amiard et Cosson (Amiard and Cosson, 1997) ont montré que ces protéines peuvent aussi être

induites par certains facteurs (stress, antibiotiques, vitamines, hormones). Selon les mêmes auteurs, la participation des MT à l'homéostasie du Zn est certaine. Cette induction est également modulée par différents facteurs tels que la condition corporelle de l'organisme cible, les rayonnements ultra-violets, la saison (Włostowski, 1992b, 1992a; Miles et al., 2000; Marques et al., 2008)… Aujourd'hui, leur induction est globalement utilisée comme marqueur d'exposition aux métaux (Brulle et al., 2007; Marques et al., 2008; Brulle et al., 2011; Manier et al., 2012). Cependant, même si leur induction est bien documentée, leur devenir après fixation des ETM chez les invertébrés, n'est pas encore bien décrit (Dallinger et al., 2004a; Dallinger et al., 2004b). Il a également été démontré que l'excrétion des métaux se liant aux MT pouvait être due à la déstructuration des MT dans les lysosomes ou à la saturation des sites de liaison des MT. Le métal se retrouvant alors sous forme libre dans l'organisme entraine soit des effets toxiques (Chabicovsky et al., 2003) ou soit une autre stratégie de détoxification comme l'excrétion (Berger et al., 1997).

Tableau 4 : Mécanismes de stockage du Cd, du Pb, de l'As, du Cr, du Cu et du Zn.

Métal	Stockage	Excrétion	Organisme étudié	Référence
Cd	fixé sur les métallothionéines et sur les granules de type B	faible	escargots	(Dallinger et al., 1993; Berger et al., 1997; Hockner et al., 2011)
Pb	fixé sur les granules de type B et C	moyenne	Invertébrés aquatiques	(Rainbow and Dallinger, 1993)
As	fixé sur les métallothionéines	faible	polychètes	(Casado-Martinez et al., 2012)
Cr	très variable, possibilité de réduction en Cr^{3+} puis fixation sur des macromolécules biologiques	très faible	Invertébrés aquatiques et poissons	(Canivet et al., 2001; Velma and Tchounwou, 2011)
Cu	fixé sur les granules de type B et métallothionéines	moyenne	Invertébrés aquatiques et escargots	(Rainbow and Dallinger, 1993; Vijver et al., 2004; Hockner et al., 2011)
Zn	Fixé sur les granules de type A et métallothionéines	moyenne	escargots	(Berger et al., 1997; Vijver et al., 2004)

L'escargot intègre de multiples sources et voies de contamination ce qui constitue un avantage car cela permet d'étudier les transferts des métaux de différents compartiments mais rend complexe l'étude des transferts de métaux *in situ*. La contribution de chaque source de contamination n'a été étudiée qu'une seule fois sur l'accumulation en laboratoire. L'influence des sources de contamination sur l'assimilation et l'excrétion n'a jamais été étudiée de même que l'influence des paramètres des sols sur cette contribution alors que ce sont eux qui modulent la mobilité des métaux.
La biodisponibilité des métaux peut être différente d'un organisme à un autre. Sa caractérisation pour de nombreux organismes dont l'escargot est donc nécessaire pour mieux la percevoir dans son ensemble. De plus, la biodisponibilité étant propre à un couple ETM-organisme, son extrapolation est difficilement réalisable d'un organisme à un autre.

V.3.c. Mesure de la biodisponibilité environnementale

- *Evaluation statique*

Pour mesurer la biodisponibilité, des mesures ponctuelles d'accumulation de métaux (bioaccumulation) peuvent être réalisées. Avec cette méthode, dite « statique », la concentration interne en métal est mesurée après une durée d'exposition définie. Cette concentration est comparée à la concentration en métal présente dans le milieu extérieur. Le rapport de la concentration interne ($mg_{métal}.kg_{esc}^{-1}$ MS) sur la concentration externe ($mg_{métal}.kg_{sol}^{-1}$ MS) représente le facteur de bioaccumulation (FBA) reflet de l'accumulation du métal par l'organisme. Cette mesure de la biodisponibilité est très utile pour une première approche comparative du comportement et du transfert de différents ETM. Cependant, L'utilisation des FBA peut être délicate. En effet, calculé sur la base des concentrations en contaminant du sol, ils n'intègrent pas les différentes sources de contamination ainsi que l'influence des paramètres des sols sur la mobilité des métaux. De plus, ces mesures ne sont qu'une image ponctuelle de la biodisponibilité qui néglige ses processus dynamiques. Cette approche ne considère que la fraction accumulée qui peut, pour les ETM excrétés, sous-estimer ce qui a réellement été assimilé. De plus, aucune information sur les concentrations internes à l'équilibre n'est fournie, paramètre pourtant important à prendre en compte afin de savoir si le risque mesuré est un risque réel ou si le risque a été sur- ou sous-estimé. Par exemple, dans le cas du Cd qui présente globalement une accumulation linéaire chez l'escargot (Gimbert et al., 2008b), la concentration à l'équilibre n'est pas forcément atteinte même après une exposition longue. Une accumulation de Cd beaucoup plus importante pourra être observée au fil du temps augmentant le risque d'apparition d'effets délétères sur les organismes contrairement au Pb avec lequel la concentration à l'équilibre en métal dans l'organisme peut être atteinte relativement rapidement (Gimbert, 2006). C'est pourquoi, il existe un besoin d'étudier la biodisponibilité des métaux sous un angle cinétique afin de posséder des informations sur les concentrations à l'équilibre dans les organismes.

- *Evaluation cinétique*

Les études statiques de biodisponibilité laissent place à un risque de mauvaise estimation des FBA de par l'absence de prise en compte des processus dynamiques de la biodisponibilité. Les cinétiques d'accumulation modélisant les patterns d'accumulation, les vitesses d'entrée des contaminants dans l'organisme et les concentrations à l'équilibre permettent d'obtenir des FBA plus fiables (Peijnenburg et al., 1999c; Van Straalen et al., 2005; Gimbert et al., 2006; Vijver et al., 2006; Gimbert et al., 2008a; Pauget et al., 2011). Par exemple, trois cinétiques théoriques d'accumulation pour trois métaux sont présentées (Figure 9, Annexe 1). On observe qu'une étude statique aurait estimé correctement le FBA uniquement pour l'ETM B (concentration à l'équilibre dans l'organisme) alors que le FBA aurait été surestimé (ETM C) ou sous-estimé (ETM A) dans les autres cas.

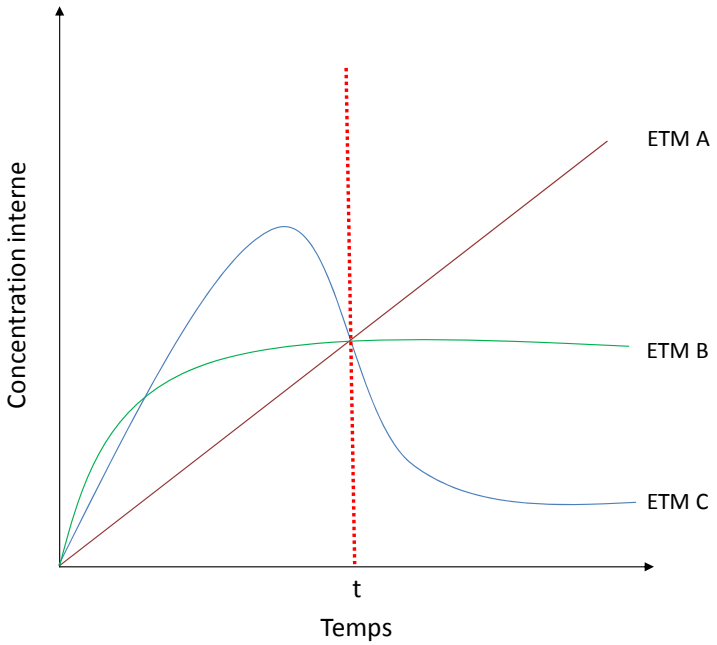


Figure 9 : Cinétiques d'accumulation théoriques pour trois ETM (A, B et C) pour un organisme exposé à un environnement contaminé. Le trait vertical rouge en pointillé représente une mesure ponctuelle des concentrations internes en ETM à un temps t. adapté de Gimbert (Gimbert, 2006).

Il existe donc un réel besoin d'estimer la biodisponibilité en tenant compte non seulement de la mobilité et de la spéciation des ETM dans les sols mais également des processus dynamiques d'accumulation.
Généralement, les études cinétiques d'accumulation prennent la forme de mesures répétées de concentrations internes au cours de l'exposition qu'il est possible de décrire par des modèles toxicocinétiques à compartiments basés sur des échanges du premier ordre entre les compartiments (Janssen et al., 1991). Le modèle utilisé au cours de cette thèse est un modèle à un compartiment (Gimbert, 2006) considérant le corps de l'escargot comme une seule unité cinétiquement homogène (Figure 10). Le modèle assume que le polluant assimilé s'équilibre rapidement et de manière homogène dans les différents fluides et tissus.

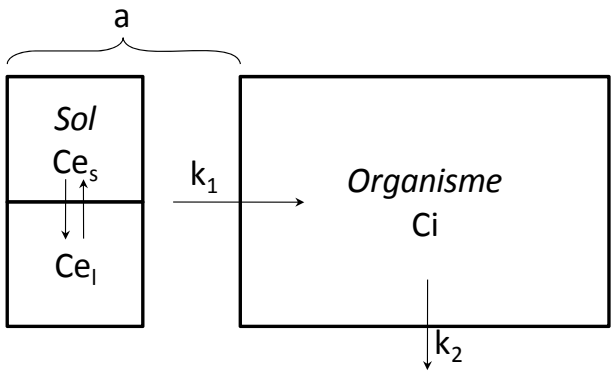


Figure 10 : Représentation schématique d'un modèle à un compartiment. Ci est la concentration interne. Ce la concentration externe biodisponible dans le sol où l'ETM est divisé en un pool métallique lié à la phase solide du sol (Ces) et un pool métallique soluble (Ce_1). a correspond au flux d'assimilation, k_1 représente le taux d'absorption et k_2 le taux d'excrétion. D'après Gimbert (Gimbert, 2006).

Le modèle cinétique retenu et présenté ci-après a été utilisé pour décrire l'accumulation des ETM chez des arthropodes (Janssen et al., 1991; Kramarz, 1999) et des vers (Peijnenburg et al., 1999c; Spurgeon and Hopkin, 1999; Vijver et al., 2005), puis a été adapté par Gimbert (Gimbert, 2006) pour l'escargot.

Ce modèle permet d'exprimer les changements dynamiques de concentration en ETM au sein de l'escargot en fonction du temps selon l'équation suivante (Eq. 1) :

(1) $$C_{esc}(t) = C_{esc}(0) + \frac{a}{k_2}\left(1 - e^{-k_2 t}\right)$$

où *a* est le flux d'assimilation ($mg_{métal}.kg_{esc}^{-1}.j^{-1}$) caractérisant la biodisponibilité en terme de vitesse d'entrée du métal dans l'organisme, étant intégrateur de l'influence des caractéristiques physico-chimiques du sol ainsi que des paramètres physiologiques sur la biodisponibilité, k_2 est le taux d'excrétion (j^{-1}), $C_{esc}(t)$ est la concentration en métal dans l'escargot ($mg_{métal}.kg\ MS_{esc}^{-1}$, MS : masse sèche) au temps *t* (j), $C_{esc}(0)$ est la concentration initiale en métal.

Pour caractériser l'accumulation des métaux dans les escargots, la concentration en métaux à l'équilibre (C_{esc} (eq)) est estimée grâce à l'équation (Eq. 2) :

(2) $$C_{esc}(eq) = C_{esc}(0) + \frac{a}{k_2}$$

A partir du flux d'assimilation *a*, un taux d'absorption k_1, paramètre cinétique caractérisant l'exposition via la voie digestive (Gimbert, 2006; Pauget et al., 2011) et reflétant l'influence des paramètres physiologiques sur la biodisponibilité peut être dérivé (Eq. 3) :

(3) $$k_{1(x)} = \frac{a}{C_x}$$

où *C* est la concentration externe supposée biodisponible et l'indice x renvoie à la méthode chimique d'estimation de la disponibilité environnementale. En fonction de la méthode utilisée et du compartiment du sol étudié (phase solide ou liquide), le taux d'absorption k_1 sera alors exprimé en $kg_{sol}.kg\ MS_{esc}^{-1}.j^{-1}$ ou $L.kg\ MS_{esc}^{-1}.j^{-1}$.

Finalement, pour caractériser le transfert de métaux du sol aux escargots, les facteurs de bioaccumulation (FBA) à l'équilibre sont calculés selon l'équation (Eq. 4) :

(4) $$FBA(eq) = \frac{C_{esc}(eq) - C_{esc}(0)}{C_x} = \frac{k_{1(x)}}{k_2}$$

La caractérisation de la biodisponibilité des métaux pour les escargots est déjà documentée. En effet, le développement de paramètres cinétiques comme le flux d'assimilation (a), le taux d'absorption (k_1) ou les FBA à l'équilibre (FBA_{eq}) ont permis d'appréhender les aspects dynamiques de la biodisponibilité des métaux pour l'escargot en particulier le Cd et le Pb (Gimbert, 2006).
Cependant, même si ces paramètres intègrent incidemment les nombreux facteurs qui modulent la biodisponibilité, il apparait nécessaire de les caractériser dans une plus large gamme de contexte d'exposition (différents sols, différentes sources…) pour gamme plus large d'ETM comme le Cu, le Sb ou l'As. Il n'existe pas de données quantifiant l'importance des caractéristiques physico-chimiques des sols sur la biodisponibilité de divers ETM pour l'escargot ou des sources de contamination selon le type de sol. On ne dispose donc pas actuellement de suffisamment de données pour proposer des modèles fiables d'évaluation du risque de transfert des ETM.

V.4. La biodisponibilité toxicologique des ETM

La biodisponibilité toxicologique correspond à la distribution de l'ETM au sein de l'organisme suite à son assimilation ainsi qu'à ses effets toxiques. Chaque organisme possédant une physiologie propre, chacun possède différentes façons de traiter un ETM une fois assimilé. Certains organismes vont excréter le plus rapidement un ETM en mettant en jeu des processus d'excrétion alors que d'autre

vont au contraire stocker les métaux dans des organes cibles comme les granules ou les MT. Pour mesurer la biodisponibilité toxicologique, la mortalité, le succès de reproduction, des biomarqueurs d'effets ou des mesures de concentration aux sites d'action toxique peuvent être utilisés et représentent ainsi la réponse de l'organisme à la toxicité de l'ETM.
Tous les effets que peuvent avoir les ETM au niveau individuel ne seront pas présentés de manière exhaustive au cours de cette thèse car ils ont été largement documentés (Vijver et al., 2004; ISO 15952, 2006; Fairbrother et al., 2007; Burger, 2008; de Vaufleury et al., 2008). De plus aux concentrations expérimentées au cours de cette thèse, aucun effet néfaste n'avait été mis en évidence.

V.5. Estimation de la biodisponibilité par la disponibilité environnementale

Pour répondre au besoin de l'ERE d'estimer la biodisponibilité des métaux à partir de méthodes chimiques de mesure de la disponibilité environnementale, la méthode chimique doit simuler les influences des paramètres du sol (pH, taux de MO, d'argile et de C_{org}, CEC, oxydes…) et de la physiologie de l'organisme sur la biodisponibilité. Pour estimer l'influence des paramètres des sols, des régressions multivariées peuvent être utilisées (Eq. 5) :

(5) $$\log(Y) = x\log(A) + y\log(B) + ,..., z$$

Où, Y correspond à la variable expliquée/mesurée (*i.e.* le flux d'assimilation (a), la concentration en métaux après 28 jours d'exposition (C_{28}), C_x …), *x, y…* représentent des coefficients et A, B … représentent les caractéristiques physico-chimiques du sol (*i.e.* concentration totale, pH, taux de MO, d'argile et de carbone organique, CEC, oxydes d'aluminium et de fer…). Le meilleur modèle est choisit sur la base du critère d'information d'Akaike corrigé (AICc, (Hurvich and Tsai, 1995; Burnham and Anderson, 2004)). L'AICc représente un compromis entre le biais, diminuant avec le nombre de paramètres libres, et la parcimonie, volonté de décrire les données avec le plus petit nombre de paramètres possibles.
Dans le but de comparer l'influence des propriétés des sols sur la disponibilité environnementale et sur la biodisponibilité des métaux, Pauget et al. (Pauget et al., 2011) ont développé une méthodologie basée sur le taux d'absorption ($k_1 = a/C_x$, Figure 11), qui prend en compte l'influence des paramètres des sols sur la disponibilité environnementale ainsi que sur la biodisponibilité environnementale dans son estimation.

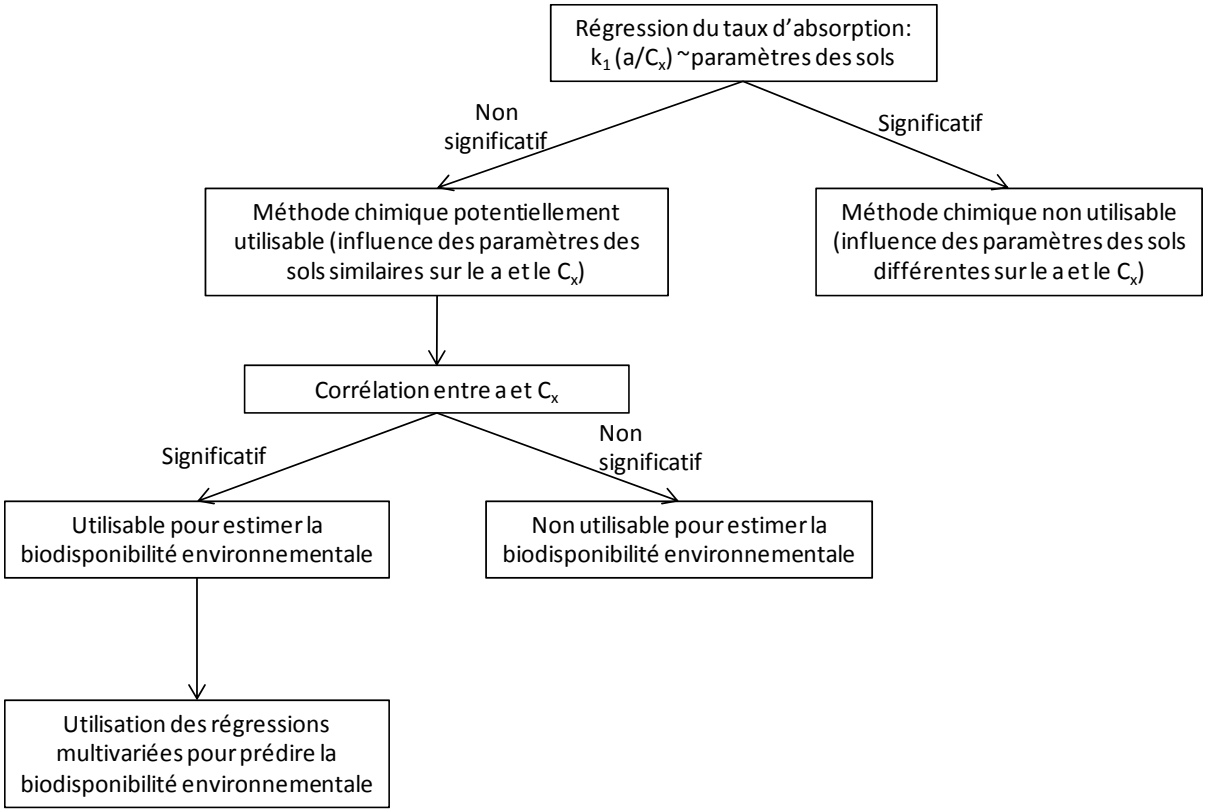


Figure 11 : Arbre de décision simplifié pour connaitre la capacité d'une méthode chimique de mesure de la disponibilité environnementale à prédire la biodisponibilité environnementale.

Nous partons du principe que lorsque les propriétés du sol influencent la disponibilité environnementale (C_x) et la biodisponibilité (a) de manière identique, la régression des taux d'absorption (k_1) en fonction des propriétés du sol ne sera pas significative (Pauget et al., 2011). Dans ce cas, la méthode chimique d'estimation de la disponibilité environnementale (C_x) pourrait être utilisée pour estimer la biodisponibilité (a) du métal pour les escargots (Figure 12).

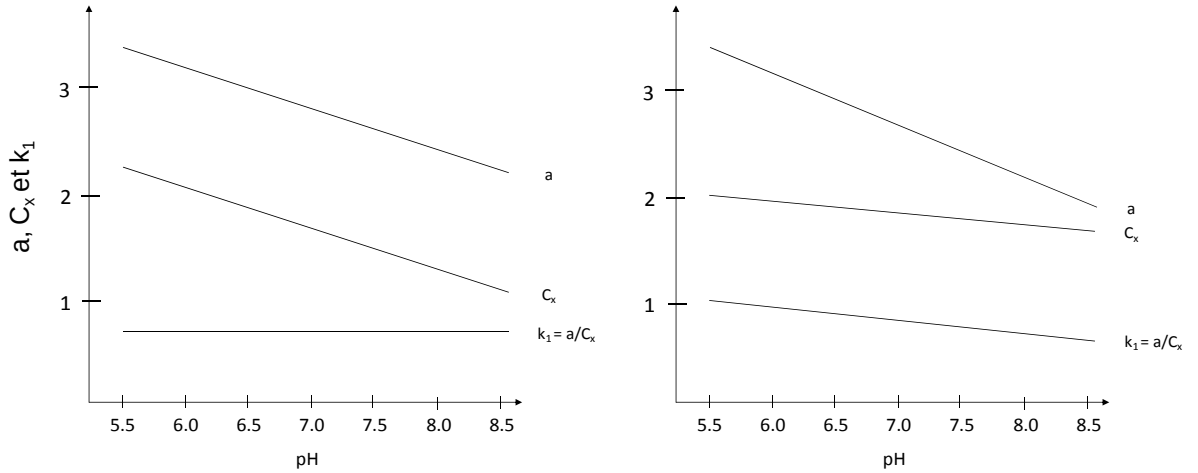


Figure 12 : Evolutions théoriques à concentration totale du sol constante du flux d'assimilation (a), de la disponibilité environnementale estimée par une méthode chimique (C_x) et du taux d'absorption ($k_1 = a/C_x$) en fonction du pH. A gauche, k_1 n'est pas dépendant du pH : le pH influence a et C_x de manière identique, C_x permet d'estimer a. A droite, k_1 est dépendant du pH : influence différente du pH sur a et C_x, C_x ne permet pas d'estimer a.

Lorsque la régression est significative (*p-value* < 0.05), la méthode chimique d'estimation de la disponibilité environnementale ne peut être utilisée pour simuler la biodisponibilité en raison de l'influence plus ou moins forte des propriétés du sol sur la disponibilité environnementale (C_x) ou sur la biodisponibilité (a) (Figure 12). Les coefficients de détermination ajustés (r^2_{adj}) de chaque régression sont utilisés pour déterminer si les principaux paramètres du sol influant sur la biodisponibilité ont été inclus dans le modèle (fort r^2_{adj}) ou si d'autres paramètres doivent être pris en compte (faible r^2_{adj}).

A la suite de cette première sélection, les capacités des méthodes chimiques à estimer la biodisponibilité des métaux pour l'escargot sont identifiées grâce au coefficient de détermination des régressions monovariées mettant en relation la biodisponibilité et la disponibilité environnementale ($a \sim C_x$).

Dans un dernier temps et dans le but de produire des équations prédictives de la biodisponibilité, les paramètres des sols sont ajoutés comme variables explicatives au C_x pour expliquer les variations de la biodisponibilité ($a \sim C_x$ + paramètres des sols).

V.6. Bilan de l'état de l'art

Cet état de l'art révèle qu'il existe des manques quant aux référentiels biologiques permettant de caractériser la contamination des sols. Pourtant un tel référentiel serait intégrateur de tous les mécanismes régissant le transfert et la biodisponibilité des métaux. Même si l'utilisation de l'escargot en tant que bioindicateur est reconnue, aucune information sur son accumulation pour certains métaux comme le Cr ou le Sb n'est disponible et pour ceux pour lesquels de nombreuses données existent, il est encore difficile de les utiliser pour établir des niveaux de référence, car les données ont été obtenues dans des conditions d'exposition disparates.

De plus, l'ERE en France se base encore sur les concentrations totales dans les sols sans tenir compte de leurs caractéristiques physico-chimiques. Ce sont pourtant ces caractéristiques qui influencent la mobilité des métaux dans les sols et incidemment leur biodisponibilité aux organismes. C'est pour prendre en compte cette influence que des méthodes chimiques d'estimation de la disponibilité environnementale dans le but de prédire la biodisponibilité des métaux pour les organismes ont été développées. Même si de nombreuses méthodes chimiques de mesure de la disponibilité environnementale ont été développées pour prédire la biodisponibilité des métaux pour les organismes, il n'existe pas à l'heure actuelle de consensus concernant une méthode chimique d'évaluation de la biodisponibilité (Scheifler et al., 2003b; van Gestel, 2008). De plus, la capacité de ces méthodes chimiques à prédire la biodisponibilité des métaux pour l'escargot *C. aspersus* n'a été réalisée que dans une seule étude en laboratoire sur des sols artificiellement contaminés qui concluait que le $CaCl_2$, pourtant recommandé par l'ISO 17402 (ISO 17402, 2008), ne permettait pas d'estimer la biodisponibilité du Cd et du Pb pour l'escargot (Pauget et al., 2011). Il apparait nécessaire de tester d'autres méthodes chimiques d'estimation du pool disponible afin de caractériser la biodisponibilité des métaux pour l'escargot.

La prise en compte de l'influence des paramètres des sols ainsi que la détermination de la contribution des différentes sources de contamination à la biodisponibilité des métaux apparait également comme nécessaire pour mieux comprendre les mécanismes modulant la biodisponibilité.

VI. Objectifs

Sur la base des lacunes identifiées dans la synthèse bibliographique, l'objectif principal de cette thèse est d'aider à la compréhension des mécanismes modulant la biodisponibilité des ETM du sol aux organismes ainsi que d'améliorer l'évaluation de l'exposition lors des procédures d'évaluation des risques actuelles en utilisant l'escargot comme bioindicateur de la pollution des sols.
Les objectifs opérationnels visent à modéliser l'accumulation et la biodisponibilité des métaux pour l'escargot en associant différentes approches tant chimiques que biologiques. Pour atteindre ces objectifs, nous avons réalisé plusieurs expériences *in situ* afin de caractériser la biodisponibilité des métaux et plusieurs expériences en laboratoire pour étudier les processus modulant cette biodisponibilité (paramètres des sols et contribution des sources).

Plus précisément, quatre objectifs sont visés au cours de ce travail dans deux contextes différents (Figure 13):

- *In situ* :

(1) Déterminer l'accumulation (mesure statique) et la biodisponibilité (mesure cinétique) des métaux du sol pour les escargots *Cantareus aspersus*.

(2) Caractériser l'influence des paramètres des sols sur l'accumulation et la biodisponibilité des métaux du sol pour les escargots *Cantareus aspersus*

Cette approche *in situ* conduira à l'élaboration d'une nouvelle méthodologie d'évaluation du risque de transfert de métaux du sol aux escargots.

- En laboratoire :

(3) Etudier la part de contamination provenant des plantes et celle provenant du sol afin d'identifier et de caractériser les contributions de ces sources de contamination pour tenter d'améliorer la modélisation des transferts de métaux aux escargots. Pour réaliser cet objectif, les flux d'assimilations des ETM provenant du sol et de ceux provenant des plantes seront étudiés. L'influence du pH et de la MO sur la contribution des sources de contamination à la biodisponibilité des métaux du sol pour les escargots *Cantareus aspersus* sera également estimée.

(4) Caractériser les paramètres du sol qui influencent la biodisponibilité des métaux en laboratoire et déterminer si une méthode chimique peut être utilisable pour simuler la biodisponibilité des métaux du sol pour l'escargot. Pour cela, l'analyse de cinétiques d'accumulation en conditions contrôlées ainsi que des mesures de la disponibilité environnementale par différents extractants permettront d'étudier les relations entre mesures chimiques et biodisponibilité.

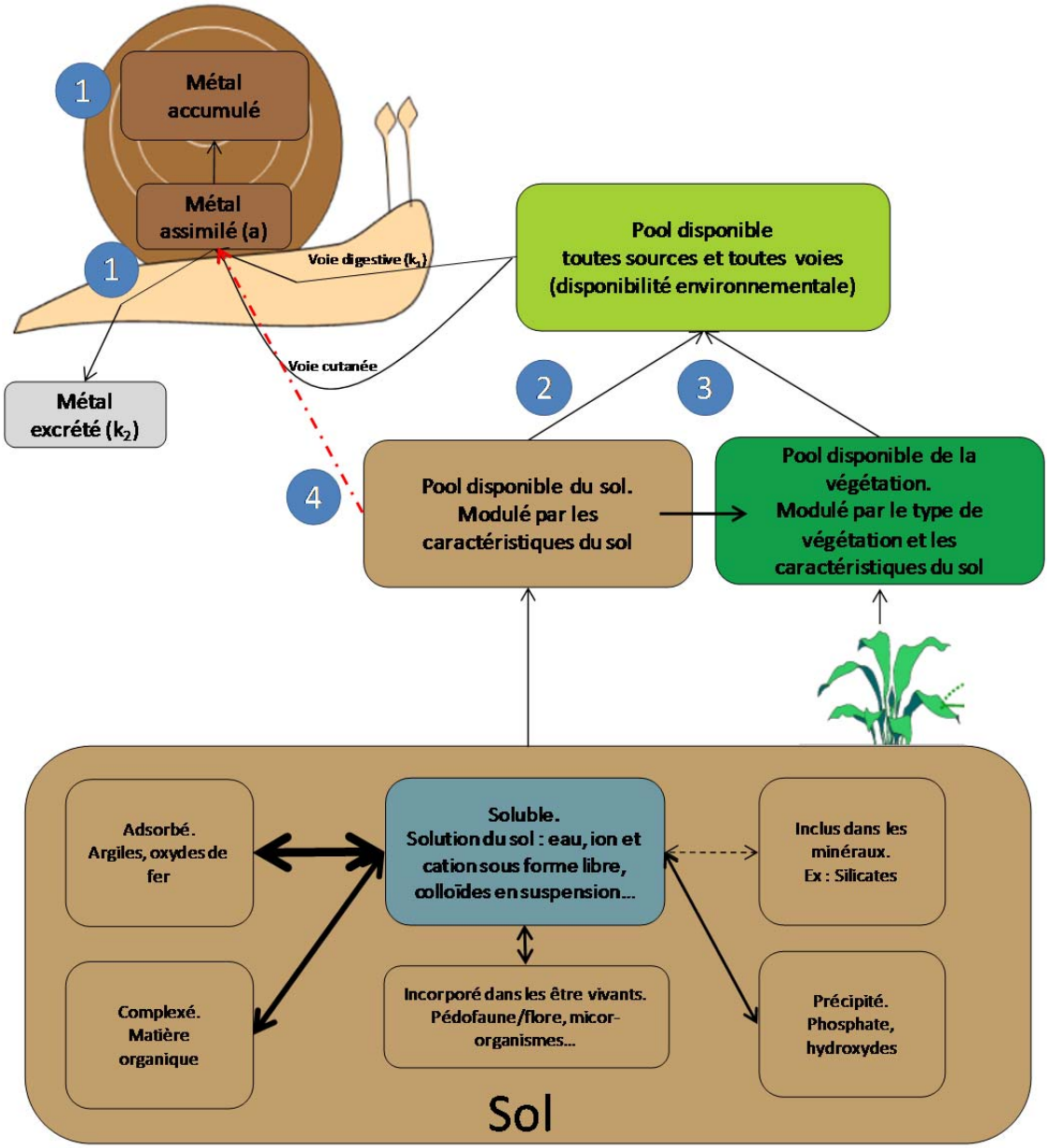


Figure 13 : Synthèse des objectifs. (1) Détermination de la biodisponibilité des métaux pour l'escargot (accumulation et flux). (2) Détermination de l'influence des paramètres du sol sur la biodisponibilité des métaux. (3) Quantification de la contribution de la source plante et de la source sol à la biodisponibilité des métaux. (4) Relation entre biodisponibilité des métaux (en terme de flux d'assimilation) et disponibilité environnementale (estimée via des mesures chimiques) (k1 : taux d'absorption (kg_{sol}.kg $MS_{esc}^{-1}.j^{-1}$ ou L.kg $MS_{esc}^{-1}.j^{-1}$), paramètre cinétique caractérisant l'exposition via la voie digestive ; (a) : flux d'assimilation ($mg_{métal}.kg_{esc}^{-1}.j^{-1}$) caractérisant la biodisponibilité en terme de vitesse d'entrée du métal dans l'organisme.

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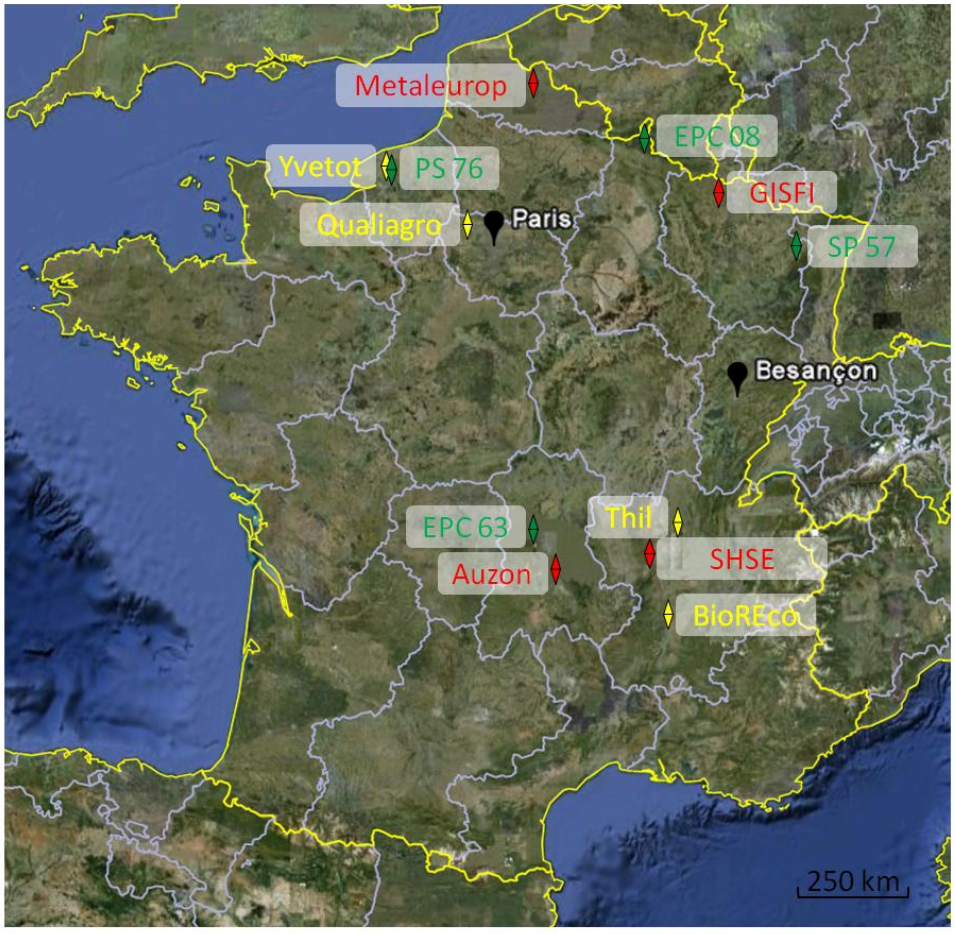
Partie B : Expérimentation *in situ*

Chapitre 1 : Studied sites

In the frame of the Bioindicator 2 program (http://ecobiosoil.univ-rennes1.fr/ADEME-Bioindicateur), twelve sites were selected including three categories (Pérès et al., 2011) (cultivated sites, forests and contaminated sites) and five land uses (forest, arable, pasture, woodland and wasteland) through whole France (Figure 14,Table 5). These sites present a large range of metal contaminations and soil parameters summarized in Table 6.

The sites of Qualiagro (Houot et al., 2002), Yvetot, BioREco and Thil, are cultivated sites and present slight metal concentrations in soils (Table 5). The forest sites, part of the RENECOFOR network (http://www.onf.fr/ renecofor) present slight metal concentrations in soils (Table 5). The sites of Auzon, Metaleurop, the slag heap of Saint-Etienne (SHSE) and GISFI are industrial sites which globally present a high soil metal pollution (Table 5).



Metaleurop : 7 plots
SHSE : 3 plots
GISFI : 2 plots
Auzon : 6 plots
Contaminated sites

Yvetot : 6 plots
Thil : 4 plots
Qualiagro : 5 plots
BioREco : 6 plots
Cultivated sites

PS 76 : 1 plot
SP 57 : 1 plot
EPC 08 : 1 plot
EPC 63 : 1 plot
Forest sites

Figure 14 : General map: localization of the studied site of the Bioindicator 2 program

Table 5: Characteristics of the 44 plots of the 12 sites of the Bioindicator program: name, plot code, land use, total content of metal elements (Metal-tot, mg/kg, ICP MS), total content of PAHs (PAH-tot, mg/kg, GC-ECD), total content of OPs (OP-tot, mg/kg, GC-ECD), total content of Herbicides (Her-tot, mg/kg, HPLC-MS/MS), texture, soil types and Description.Texture: Silt (Si), Silty clay Loam (SiCL), Loam (L), Silty Loam (SiL), Loamy Sand (LSa), Sand (Sa), Sandy clay Loam (SaCL), Loamy clay Sand (LCSa), Clay sandy Loam (CSaL), Clay sand (CSa). Soil type (Baize and Girard, 2008): Antroposol (AN), Fluvisol Redoxisol carbonated (FV), Brunisol Redoxisol (BR), Fluviso (FU), Brunisol (B), Technosol (Tec), Luvisol (LU), Neo-Luvisol (NLU), Stony Fluvisol (SFU), Podzol (PO), Alocrisol (AL), Andosol mollique (AM). For quantitative value, the median value is used.

Site name	Plot Code	Land use	Metal-tot	PAH-tot	OP-tot	Her-tot	Texture	Soil type	Description
GISFI	GHF	Wasteland	779.0	60.5	1.8	808.1	LSa	AN	Low PAH contamination, wasteland
	GHM	Wasteland	990.5	572.2	1.8	17850.0	LSa	AN	Medium PAH contamination, wasteland
Metaleurop	HW	Woodland	4597.1	2.0	7.8	2285.3	SiCL	FV	High metal contamination, woodland
	IW	Woodland	1626.8	0.5	5.4	6395.6	SiCL	FV	Intermediated metal contamination, woodland
	IA	Arable	1149.2	0.9	1.3	6502.3	SiCL	FV	Intermediated metal contamination, arable
	LW	Woodland	764.9	1.7	1.2	8180.7	L	BR	Low metal contamination, woodland
	LA	Arable	466.5	1.0	1.8	3191.8	L	BR	Low metal contamination, arable
	RW	Woodland	234.4	0.4	7.1	1864.6	SiL	BR	Reference woodland site
	RA	Arable	228.2	0.3	0.8	3017.6	SiL	BR	Refence arable site
Auzon	CoWW	Woodland	8399.5	10.0	5.6	887.0	SaCL	FU	Contaminated woodland on hydromorphic soil
	CoW	Woodland	723.8	5.2	2.5	279.6	LCSa	FU	Contaminated woodland
	CoWH	Woodland	1246.3	7.7	0.6	697.0	Lsa	FU	Contaminated woodland hedge
	CoWa	Wasteland	3344.5	2.9	0.9	456.5	Lsa	FU	Contaminated wasteland
	CtW	Woodland	466.6	3.2	2.4	9.4	LCSa	FU	Control woodland
	CtWH	Woodland	301.4	0.2	0.0	766.4	Csa	BR	Control woodland hedge
	CtP	Pasture	397.9	2.3	1.1	764.8	LSa	BR	Control pasture
SHSE	HCV	Wasteland	9493.2	31.6	2.0	82005.2	Sa	Tec	High level of cover of vegetation (with approx. 60-70% of plant cover)
	ICV	Wasteland	6375.8	20.1	1.9	25152.6	Sa	Tec	Intermediate level of cover of vegetation (with approx. 30% of plant cover)
	LCV	Wasteland	7830.3	5.8	0.0	5915.6	Sa	Tec	Low level of cover of vegetation (with 5-10% of plant cover).
QualiAgro	Ct	Arable	162.0	0.3	18.0	2138.1	Si	LU	Control
	FYM	Arable	179.0	0.4	28.0	2028.5	Si	LU	farmyard manure

Table 5 (cont.)

Site name	Plot Code	Land use	Metal-tot	PAH-tot	OP-tot	Her-tot	Texture	Soil type	Description
QualiAgro	BIOW	Arable	173.9	0.3	25.0	1305.4	Si	LU	biowaste compost issued from the co-composting of green wastes and source-separated organic fractions of municipal solid wastes
	GWS	Arable	178.7	0.4	25.0	2939.4	Si	LU	compost issued from the co-composting of green wastes with sewage sludge
	MSW	Arable	180.0	0.3	18.0	1334.8	Si	LU	a municipal solid waste compost issued from the composting of residual solid wastes after removing dry and clean packaging
Yvetot	RP1	Arable	170.1	0.2	1.0	760.0	CSaL	NLU	Rotation pasture (2 yr of pasture) restored after a cropping period of 5yr
	RP2	Arable	170.0	0.2	1.3	524.6	CSaL	NLU	Rotation pasture (2 yr of pasture) restored after a cropping period of 6yr
	TP2	Arable	177.8	0.1	0.0	435.4	CSaL	NLU	Temporary pasture (implanted with wheat at the sampling time) from an arable (2 yr maize-wheat rotations) and grassland (4-8yr) rotation management.
	TP1	Arable	167.2	0.2	4.5	268.4	CSaL	NLU	Temporary pasture (5 yr of pasture at sampling time) from an arable (2 yr maize-wheat rotations) and grassland (4-8yr) rotation management.
	PP	Arable	179.9	0.5	0.6	81.6	CSaL	LU	Permanent Pasture (> 25 yr) with permanent cover and no tillage
	GC	Arable	154.0	0.4	0.0	81.2	CSaL	NLU	Long-term arable crop (10 yr) with intensive management (ploughing/fertilization)
BioREco	OG_A	Orchard	200.8	1.4	4.7	5919.6	Csa	SFU	Organic farming system (no synthetic inputs) with "Ariane" cultivar
	OG_GD	Orchard	176.5	0.1	7.7	713.3	LCSa	SFU	Organic farming system (no synthetic inputs) with "Golden Delicious" cultivar
	LI_A	Orchard	190.7	0.1	6.1	1024.0	LCSa	SFU	Low Input system (low chemical input) with "Ariane" cultivar
	LI_GD	Orchard	209.2	0.1	0.4	2489.6	LCSa	SFU	Low Input system (low chemical input) with "Golden Delicious" cultivar
	CV_A	Orchard	183.2	0.1	0.3	2845.8	LCSa	SFU	Conventional system (use of pesticides) with "Ariane" cultivar
	CV_GD	Orchard	195.0	0.1	0.3	1568.4	LCSa	SFU	Conventional system (use of pesticides) with "Golden Delicious" cultivar
Thil	ST	Arable	143.7	0.0	0.2	216.8	Csa	FU	Superficial Tillage (less than 5 cm depth)
	SMP	Arable	143.9	0.0	0.2	303.5	Csa	FU	Shallow Mouldboard Ploughing, 20 cm depth
	MP	Arable	119.3	0.0	0.2	168.4	Csa	FU	Traditional Mouldboard Ploughing, tilling to a depth of 30 cm
	RT	Arable	135.5	0.0	0.5	187.9	Csa	FU	Reduced Tillage, with tined tools (15 cm depth)
RENECOFOR	F08	Forest	224.3	0.8	1.0	77.2	L	PO	Spruce forest
	F57	Forest	43.9	0.1	0.4	569.3	Sa	AL	White fir forest
	F76	Forest	66.1	0.5	4.1	569.3	LSa	PO	Scots pine forest
	F63	Forest	285.3	0.3	2.7	457.2	SiCL	AM	Spruce forest

Table 6: Medians of physico-chemical characteristics of the 12 Bioindicator program sites studied.

Site	Plot	Soil														
		pH	Silts ($g\ kg^{-1}$)	Sands ($g\ kg^{-1}$)	OM ($g\ kg^{-1}$)	Clay ($g\ kg^{-1}$)	CEC ($cmol\ kg^{-1}$)	C_{org} ($g\ kg^{-1}$)	$[Al]_{ox}$ ($cmol\ kg^{-1}$)	$[Fe]_{ox}$ ($cmol\ kg^{-1}$)	$[Cd]_{tot}$ ($mg\ kg^{-1}$)	$[Pb]_{tot}$ ($mg\ kg^{-1}$)	$[As]_{tot}$ ($mg\ kg^{-1}$)	$[Cr]_{tot}$ ($mg\ kg^{-1}$)	$[Cu]_{tot}$ ($mg\ kg^{-1}$)	$[Zn]_{tot}$ ($mg\ kg^{-1}$)
GISFI	GHF	8.30	225	611	209	143	15.0	121	0.067	0.019	0.499	165	32.8	57.1	30.3	408
	GHM	8.33	172	736	363	94.0	11.0	210	0.026	0.024	1.23	309	58.5	171.50	45.3	323
Metaleurop	HW	7.99	601	101	83.5	298	29.3	48.3	0.038	0.014	34.4	2485	39.3	48.6	68.4	1885
	IW	8.19	609	96.0	46.0	294	24.3	26.6	0.040	0.020	13.3	731	30.9	50.3	27.6	745
	IA	8.21	474	211	45.1	318	29.3	26.1	0.054	0.014	8.54	482	18.1	61.5	28.7	509
	LW	6.45	283	517	55.0	203	17.2	31.8	0.059	0.015	5.44	319	9.42	52.0	19.4	332
	LA	7.90	366	452	29.3	181	13.2	16.9	0.078	0.020	3.12	143	8.67	49.4	15.1	226
	RW	6.55	525	313	34.9	163	12.2	20.2	0.053	0.011	1.09	48.8	7.11	41.5	12.3	102
	RA	6.96	531	290	25.5	181	13.0	14.7	0.048	0.010	0.968	41.7	8.60	45.1	14.2	92.0
Auzon	CoWW	5.41	427	346	103	238	19.1	59.6	0.425	0.015	9.97	4575	3285	76.5	159	225
	CoW	5.73	279	574	70.6	148	13.0	40.8	0.176	0.016	0.722	104	339	57.9	22.9	148
	CoWH	5.23	167	757	76.5	89.5	11.0	44.2	0.410	0.017	1.32	282	661	51.8	38.6	140
	CoWa	5.81	167	713	60.3	98.5	9.46	34.9	0.111	0.009	6.74	1834	1087	52.3	140	173
	CtW	6.22	299	556	70.7	138	14.9	40.9	0.069	0.012	0.612	60.1	123	67.3	27.7	138
	CtWH	5.10	225	628	45.2	148	8.90	26.2	0.814	0.016	0.143	28.1	62.5	56.8	22.8	88.1
SHSE	HCV	8.10	142	803	81.1	48.5	10.5	46.9	0.081	0.030	21.0	2525	73.3	982	1555	2830
	ICV	8.74	131	820	52.5	44.5	6.60	30.4	0.065	0.026	9.37	1616	54.8	1158	570	2180
	LCV	8.57	77	886	25.6	33.5	3.47	14.8	0.070	0.031	1.99	513	30.4	4345	525	577
Qualiagro	Ct	6.84	783	66.5	17.7	156	8.42	10.2	0.072	0.015	0.206	22.1	7.36	47.7	12.0	50.1
	FYM	7.46	777	67.0	24.4	156	9.74	14.1	0.069	0.014	0.226	26.7	7.22	47.4	15.8	58.4
	BIOW	7.81	771	68.5	25.2	161	10.50	14.6	0.080	0.016	0.230	25.6	7.83	45.0	14.0	57.7
	GWS	7.00	780	72.0	26.3	151	9.60	15.2	0.078	0.016	0.222	24.2	6.77	48.6	16.4	59.0
	MSW	7.62	774	71.0	21.5	155	9.64	12.4	0.070	0.014	0.231	28.3	7.26	47.0	15.2	58.9
Yvetot	RP1	5.58	665	196	19.2	140	5.43	11.1	0.208	0.008	0.236	21.0	6.93	66.3	10.3	42.7
	RP2	6.03	665	210	24.8	128	6.97	14.4	0.066	0.009	0.243	30.6	6.76	49.0	14.4	47.0
	PT2	6.25	655	204	23.9	142	7.74	13.8	0.058	0.010	0.217	27.9	8.57	51.3	14.8	50.7
	PT1	5.50	652	202	32.6	149	7.25	18.9	0.172	0.007	0.205	21.4	8.52	51.2	13.7	48.4
	PP	5.48	634	203	44.6	163	8.06	25.8	0.255	0.008	0.198	26.3	9.48	53.8	13.4	51.5
	GC	6.42	670	197	18.6	132	7.02	10.8	0.050	0.009	0.199	19.0	8.14	49.2	10.5	45.4
BioREco	OG_A	7.13	261	533	21.4	204	9.39	12.3	0.110	0.023	0.147	23.7	12.3	53.6	24.4	47.4
	OG_GD	7.10	292	556	18.4	158	6.93	10.6	0.106	0.021	0.164	22.4	9.36	44.7	26.3	41.8
	LI_A	7.35	286	528	24.9	186	9.39	14.4	0.139	0.027	0.148	26.5	10.6	48.2	19.5	46.6
	LI_GD	7.28	293	520	22.9	184	8.56	13.3	0.094	0.018	0.165	29.3	11.4	48.9	32.2	49.3
	CV_A	6.89	297	540	23.9	162	6.91	13.8	0.082	0.016	0.182	31.1	10.4	46.4	19.8	42.2
	CV_GD	7.36	296	545	25.1	164	8.61	14.5	0.095	0.018	0.178	26.2	10.3	47.6	31.9	42.9
Thil	ST	8.13	235	617	27.7	143	9.78	16.0	0.036	0.007	0.212	14.6	6.55	43.3	8.22	41.9
	SMP	8.23	224	641	23.5	130	8.91	13.6	0.061	0.012	0.227	14.7	6.53	44.8	8.91	42.3
	MP	8.28	181	720	19.8	100	6.59	11.4	0.050	0.010	0.189	12.8	5.67	36.5	7.57	35.3
	RT	8.20	212	673	21.9	124	8.39	12.6	0.056	0.010	0.209	14.3	6.56	43.4	8.92	40.1
RENECOFOR	F08	4.05	630	106	151	262	10.4	87.4	7.00	0.126	0.235	78.9	25.4	57.9	17.1	34.0
	F57	4.13	73.0	886	22.5	44.5	2.14	13.0	1.43	0.027	0.025	21.1	3.83	6.55	1.73	8.62
	F76	3.77	354	566	180	78	7.10	104	1.87	0.059	0.063	21.9	2.91	17.7	3.48	13.5
	F63	4.87	411	326	269	257	9.42	156	5.64	0.046	0.414	49.7	12.8	55.0	14.1	113

I. Cultivated sites

I.1. Qualiagro

I.1.a. Site description

The Qualiagro experiment (a cultivated site, code FEU) is located at Feucherolles, in north-western France (50 km west from Paris; 48°52’ N, 1°57’ E). The climate is oceanic with a mean rainfall of 695 mm year–1 113 and a mean annual temperature of 10.7°C. The soil is cultivated following a wheat-maize rotation. The organic amendments are applied every two years in September on wheat stubble. Since the beginning of the experiment (1998), 6 applications have been applied in 1998, 2000, 2002, 2004, 2006 and 2007.

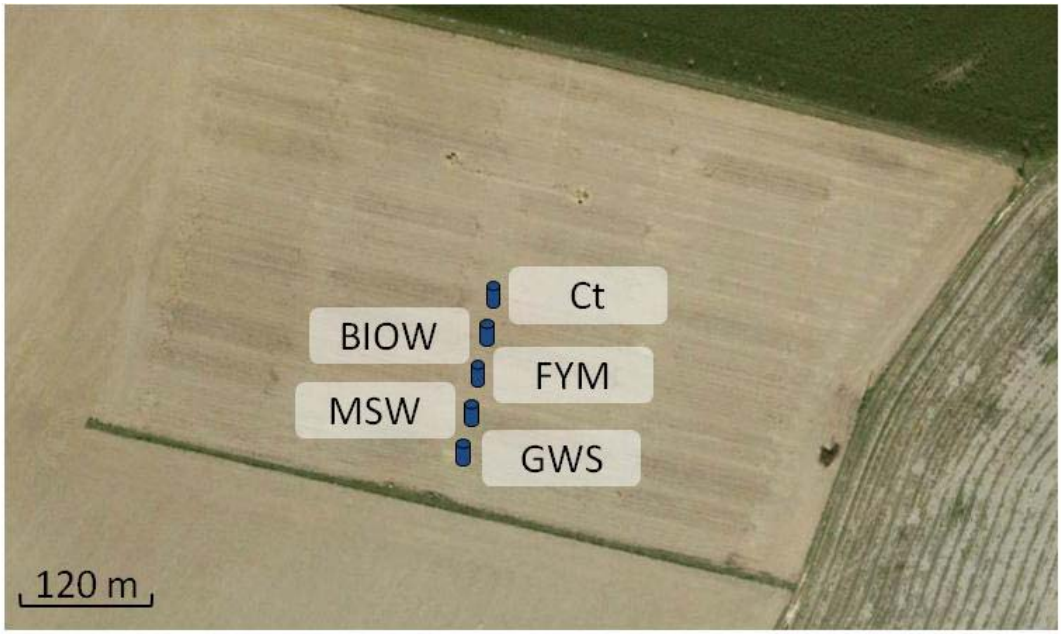


Figure 15: Localization of the microcosms on the plots of the Qualiagro site. GWS: green wastes with sewage sludge, MSW: municipal solid wastes, FYM: farmyard manure, BIOW: biowaste compost, Ct: control. A blue cylinder represents a microcosm.

I.1.b. Plot description

Five plots were selected (Figure 15): four types of organic amendments are compared to a control treatment (Ct): (i) a biowaste compost (BIOW) issued from the co composting of green wastes and source-separated organic fractions of municipal solid wastes, (ii) a compost (GWS) issued from the co-composting of green wastes with sewage sludge, (iii) a farmyard manure (FYM), and (iv) a municipal solid waste compost (MSW) issued from the composting of residual solid wastes after removing dry and clean packaging (Figure 16).

Figure 16: Snails exposure in microcosms on the Qualiagro site.

I.2. Yvetot

I.2.a. Site description

The Yvetot site is an agricultural site on which different crop/pasture management practices are tested (Plassart et al., 2008). This site is located in Normandy (49°36'N, 0°44'E, North-western France, 35 km north-west from Rouen) and extends around the agricultural college of Yvetot. The soil texture is classified as silt loam (Luvisol) soil in all plots containing 15% clay, 65% silt, and 20% sand. The climate is dominated by an oceanic and temperate climate with a mean of rainfall of 844 mm $year^{-1}$ and a mean annual temperature of 11,1°C. All pastures were implanted with perennial ryegrass and clover.

I.2.a. Plots description

Six plots were selected (Figure 17): one arable cropping (GC), two rotation pastures (RP1, RP2) which correspond to restored pastures after 5-6 yr of cropping period, two temporary pastures (TP1, TP2) which correspond to different arable-grassland rotations, and one permanent pasture (PP) (Figure 18).

Figure 17: Localization of the microcosms on the plots of the Yvetot site. RP: rotation pasture 1 and 2, TP: temporary pasture 1 and 2, PP, permanent pasture, GC: culture. A blue cylinder represents a microcosm.

RP1

TP1

RP2

PP

TP2

GC

Figure 18: Snails exposure in microcosms on the plots of the Yvetot site. RP: rotation pasture 1 and 2, TP: temporary pasture 1 and 2, PP, permanent pasture, GC: culture.

I.3. BioREco

I.3.a. Site description

The BioREco site is an experimental site from INRA created in 2005 (Simon et al., 2011) located in INRA research unit Gotheron (44°97'N, 4°92 E), in the Middle Rhone valley (France). It covers a surface of 3.3 ha. The apple orchard was assumed to have no drainage and no slope is considered, with relatively dry weather during summer period due to Mediterranean influences and around 850 L/m^2 per year of rainfall. Planting density is 1000 trees/ha, with a grass cover between rows. The soil contains 2% humus, 15% clay and the rooting depth is at around 40 cm. Each plot (0.37ha), referred to as 'production system' (or 'system' in short), is a combination of one farming system and one apple cultivar. Three farming systems were tested: (i) in Conventional system (CV), chemical pesticides were mainly used to control pests, diseases and weeds; bulletin of extension services were used to schedule treatments; (ii) in Organic Farming (OG), no synthetic inputs were applied, as defined by the European rules for organic production (EEC 91/2092); (iii) in Low-input system (LI), preference was given to other protection methods than chemicals (mating disruption, sanitation practices…). Two planted cultivars were also tested: (i) Smoothee 2832T®: a 'Golden Delicious' type cultivar susceptible to scab, a major fungal disease due to *Venturial inaequalis*. Smoothee 2832T® is referred to as 'Golden D; (ii) Ariane: scab resistant (*Vf*-gene).

Figure 19: Localization of the microcosms on the plots of the BioREco site. OG: organic farming system, LI: low input system, CV: conventional system, GD: "Golden Delicious" cultivar, A: "Ariane" cultivar. A blue cylinder represents a microcosm.

I.3.b. Plot description

Six plots were selected (Figure 19), combining three different protection strategies (conventional supervised (CV), low input (LI) and organic farming (OG)) and two apple cultivars with different susceptibility to disease: "Ariane" susceptible to scab (A) and "Golden Delicious" resistant to scab (GD) (Figure 20).

CV_A

LI_A

CV_GD

OG_A

LI_GD

OG_GD

Figure 20: Snails exposure in microcosms on the plots of the BioREco site. OG: organic farming system, LI: low input system, CV: conventional system, GD: "Golden Delicious" cultivar, A: "Ariane" cultivar.

I.4. Thil

I.4.a. Site description

The Thil site is an agricultural site managed in organic farming, on which different tillage managements are compared since 2005 (Vian et al., 2009) located near Lyon (45°49' N, 5°22 E, France). It has been managed in organic farming since 1999. The soil is a Fluvisol. The climate is continental with degraded oceanic conditions (730 mm of mean rainfall and 10.7 °C of mean temperature). The four tillage systems were compared using the normal organic farming methods, with a traditional crop rotation for the region: alfalfa (3 years) / maize / soya / winter wheat / soya / winter wheat associated with an undersown alfalfa during the spring / maize.

Figure 21: Localization of the microcosms on the plots of the Thil site. SMP: Shallow Mouldboard Ploughing, RT: Reduced Tillage, ST: Superficial Tillage, MP: Traditional Mouldboard Ploughing. A blue cylinder represents a microcosm.

I.4.b. Plot description

Four plots were selected (Figure 21): (i) Traditional Mouldboard Ploughing (MP), (ii) Shallow Mouldboard Ploughing (SMP), (iii) Reduced Tillage (RT) with tined tools and (iv) Superficial Tillage (ST) (Figure 22).

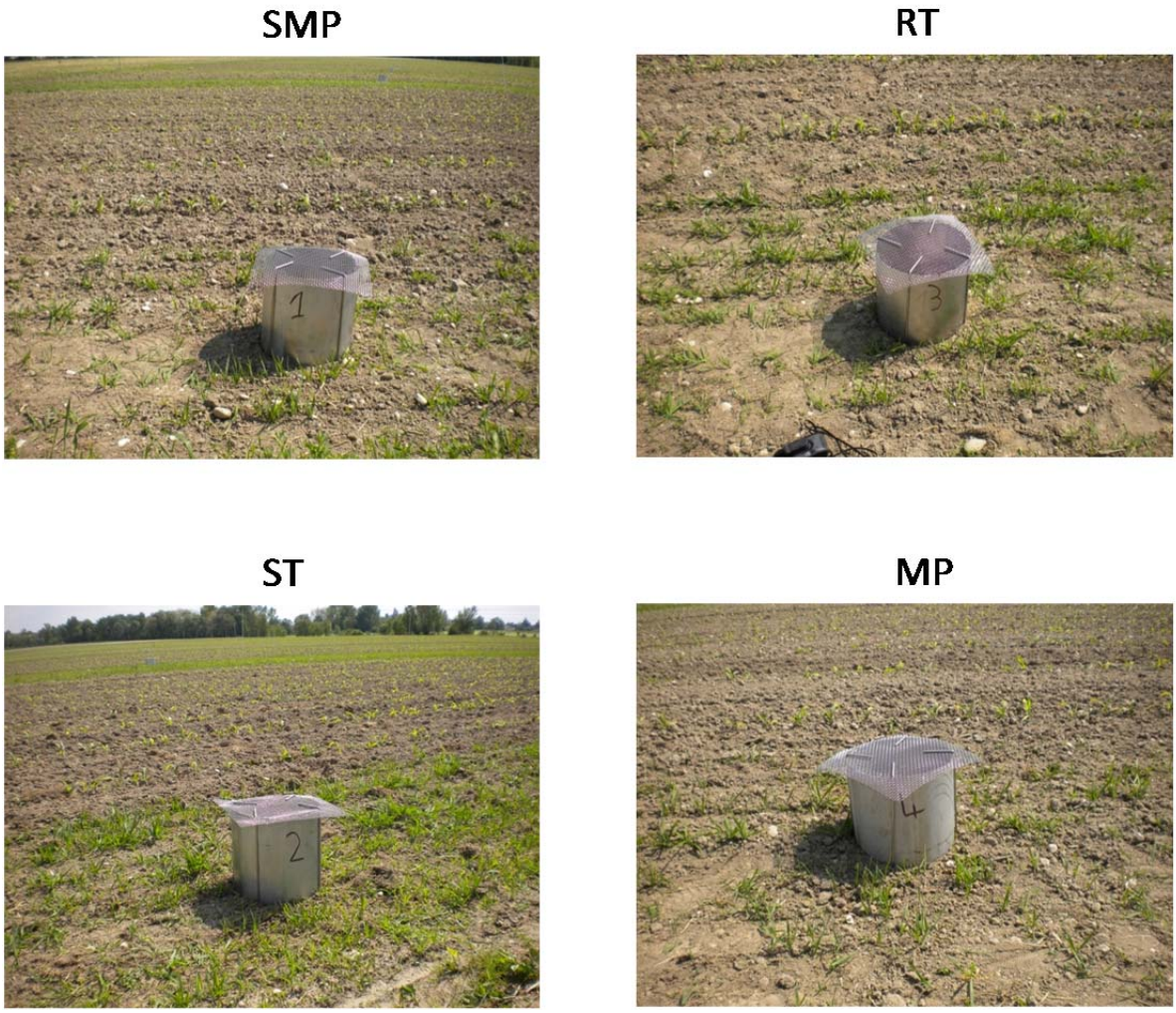


Figure 22: Snails exposure in microcosms on the plots of the Thil site. SMP: Shallow Mouldboard Ploughing, RT: Reduced Tillage, ST: Superficial Tillage, MP: Traditional Mouldboard Ploughing.

II. Forest sites

II.1. Site description

Four forest sites, which were part of the RENECOFOR network (http://www.onf.fr/renecofor), were studied. ECP08 site, a common spruce forest , is located in the Croix-Scaille forest in northern France (49°56'N, 4°48'E). The climate is semi-oceanic with a mean rainfall of 1090 mm year^{-1}, a mean temperature of 10.8°C and the altitude is 470 m with an average slope of 6%. Geological substrate is schist and soil type is a podzol (Baize and Girard, 2008) with loamy texture. The humus type is Moder. SP57 site, a white fir forest, is located in the Abreschviller forest in eastern France (48°36'N, 7°8'E). The climate is continental with mean rainfall of 785 mm year^{-1}, a mean temperature of 9°C and the altitude is 400 m with an average of slope 26%. Geological substrate is sandstone and soil type is an alocrisol (Baize and Girard, 2008) with a sandy. The humus type is Mull. The PS76 site is a scots pine forest located in the Brotonne forest, in Haute Normandie region in north-western France (49°45'N, 0°74 E). The climate is dominated by an oceanic and temperate climate with a mean of rainfall of 877 mm year^{-1} and a mean annual temperature of 10.5°C. The altitude is 70 m with an average slope of 3%. The geological substrate is sandy-loam with flint and the soil type is a Podzol

(Baize and Girard, 2008) with loamy sandy texture. The humus type is Mor. The F63 forest site is a common spruce forest, located in the Manson forest, at Saint-Genès Champanelle (Puy de Dôme) in center of France (45°75'N, 2°96'E). The climate is continental with an oceanic tendency (the mean rainfall is 993 mm.an^{-1} and a mean temperature was 7.7°C) and the altitude is 950 m with an average slope of 1%. The geological substrate is basalt and the soil type is an Andosol (Baize and Girard, 2008) with loamy sandy texture. The humus type is DysMull.

II.2. Plot description

On each forest site, one plot was selected (Figure 14): F08 on the EPC08 site, F57 on the SP57 site, F76 on the PS76 site and F63 on the EPC63 site (Figure 23).

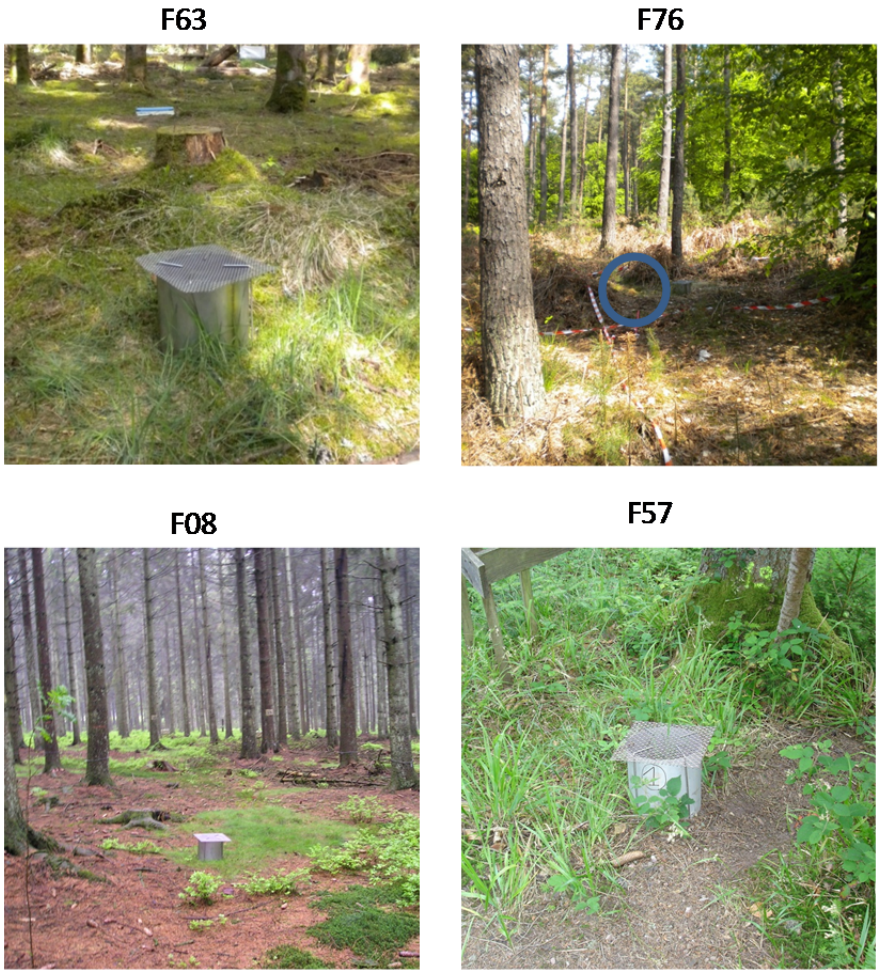


Figure 23: Snails exposure in microcosms on the plots of the forest site.

III. Contaminated sites

III.1. Auzon Site

III.1.a. Site description

The Auzon site (45°23'12N, 03°21'32 E) is an industrial waste site presenting a trace metal contamination such as arsenic located in the Massif Central region, France. Listed by the French Ministry of the Environment as one of the most polluted sites in France, it is a former factory where

pesticides and fertilizers responsible for trace metal contamination, and notably for As, were manufactured and stored. The entire landfill covered an area of about 1 ha. The climate is continental with an oceanic tendency (the mean rainfall was 800 mm.an^{-1} and a mean temperature was 10.8°C). Industrial activities started at the beginning of the 20th century and lasted in 1949. The trace metal elements pollution in soils was dominated by As, Pb ans Sb with a high spatial heterogeneity (Laperche and Eisenlohr, 2001). Control areas were located approximately 2 km from the contaminated site (Figure 24).

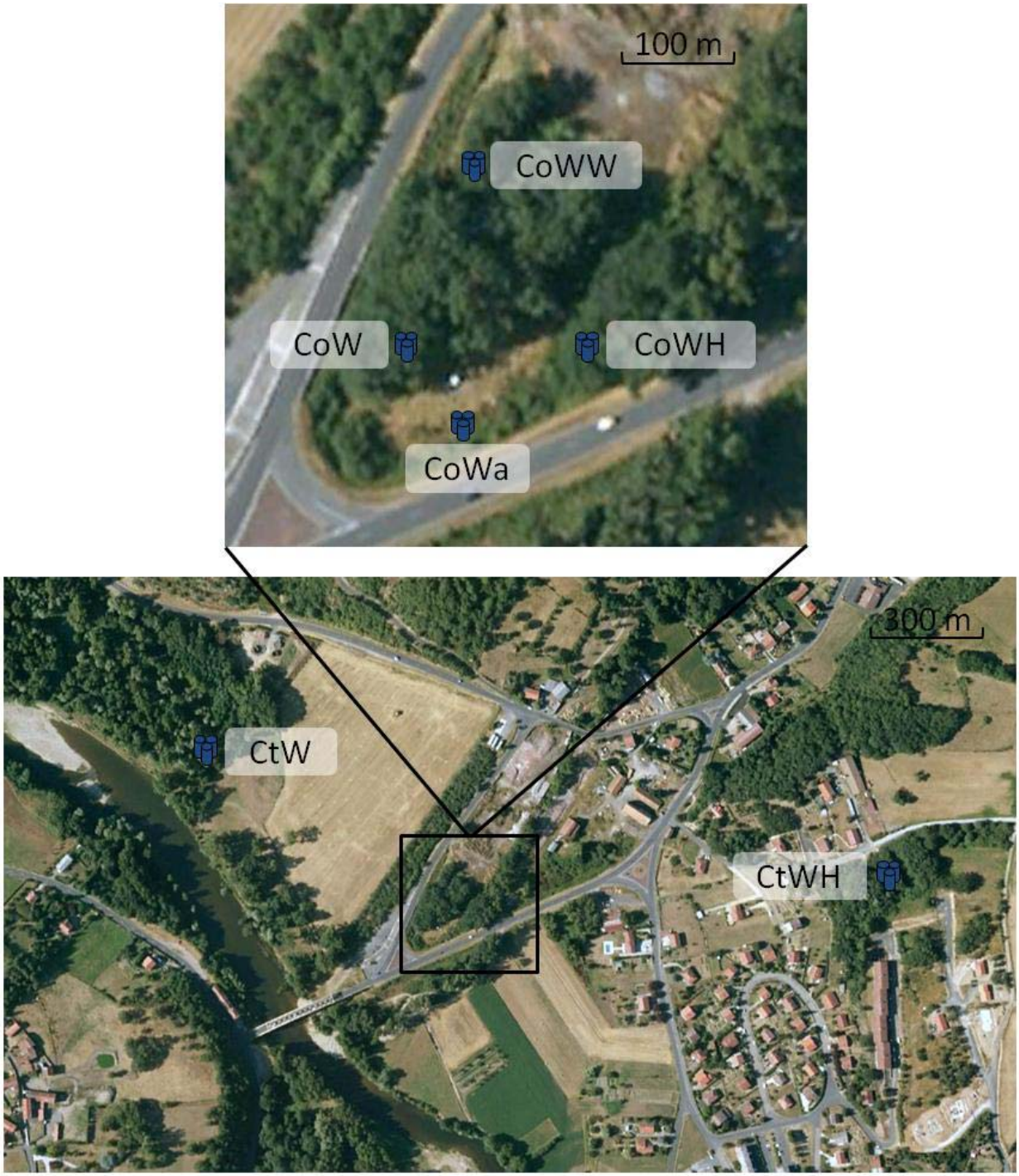


Figure 24: Localization of the microcosms on the plots of the Auzon site. CtW: control woodland, CtWH: control woodland hedge, COWa: contaminated wasteland, CoWH: contaminated woodland hedge, CoWW, contaminated wet woodland, CoW: contaminated woodland. A blue cylinder represents a microcosm.

III.1.b. Plots description

Six plots (4 contaminated “Co”, 2 controls “Ct”) were selected according to a metal pollution gradient (from 62 to 3600 $mg.kg^{-1}$ As) and vegetal cover (woodland “W”, woodland on hydromorphic soil “WW”, woodland hedge “WH” and wasteland “Wa”) (Figure 25).

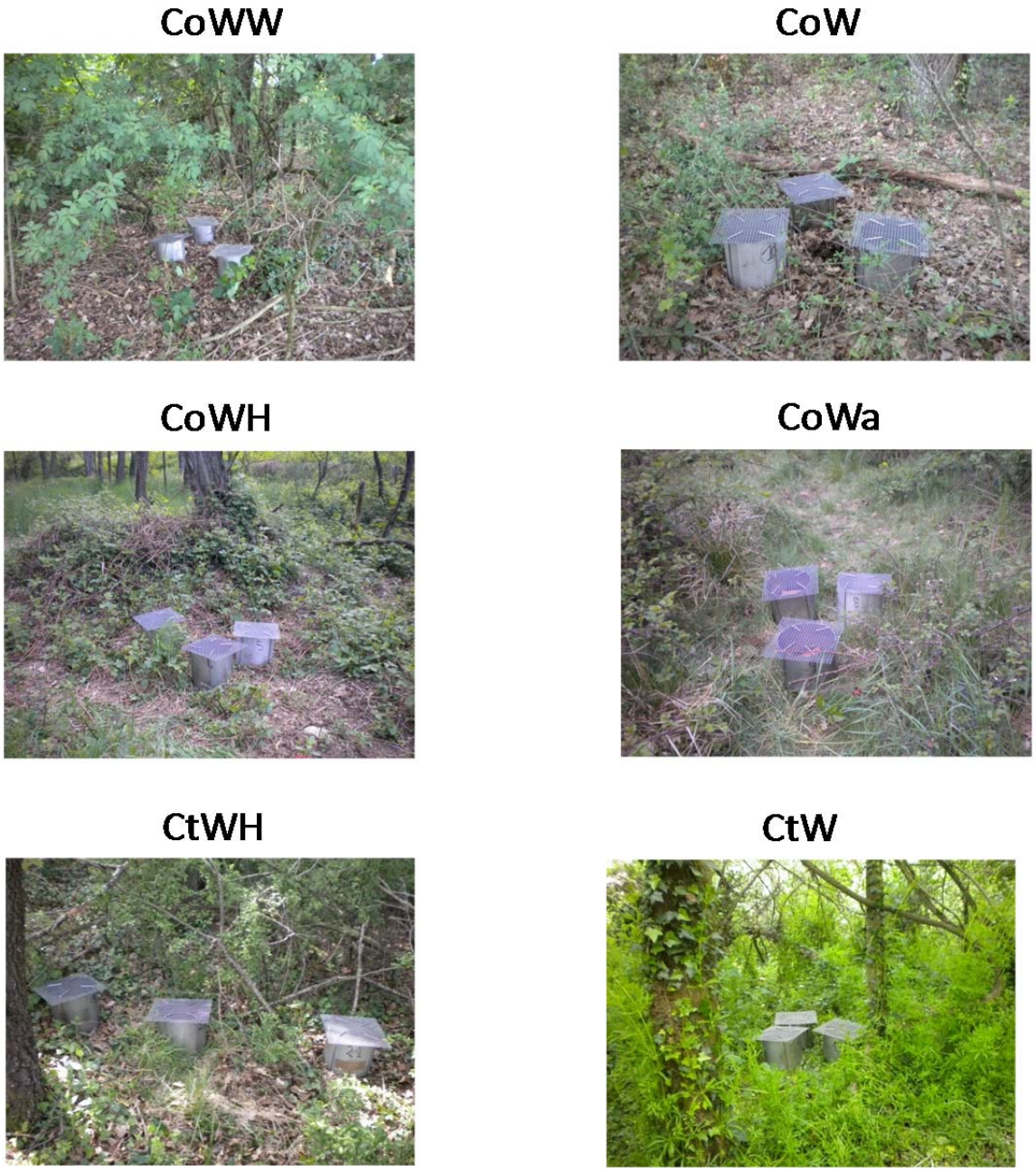


Figure 25: Snails exposure in microcosms on the plots of the Auzon site. CtW: control woodland, CtWH: control woodland hedge, COWa: contaminated wasteland, CoWH: contaminated woodland hedge, CoWW, contaminated wet woodland, CoW: contaminated woodland.

III.2. The Slag heap of Saint-Etienne (SHSE)

III.2.a. Site description

The SHSE site is a metallurgical landfill near St-Etienne (45°43'N, 4°39'E), presenting a high metal contamination combined with herbicide contamination. It has been used from about 1850 to 2001 to dispose of foundry waste, and extend over nearly 15 ha. It is under a temperate continental climate, at an altitude of 250 m. Mean annual temperature is 10 °C, with minimal and maximal values ranging from -26 °C to + 41 °C. Annual precipitation averages 750 mm. The soil at this site was not originated from the underlying bedrock but was derived from the progressive transformation of the waste deposits, resulting in a metalliferous technosoil. Although the studied area had remained undisturbed since several years, the vegetation cover was not homogenous: some plots were not or very scarcely colonized by plants, while other showed relatively high plant diversity. Therefore, this cover heterogeneity led in the selection of the three plots according to their level of plant cover. These three plots were highly contaminated with various metals, mainly Cd, Cr, Cu, Mo, Ni, Pb and Zn; they were also contaminated with some herbicides (mostly Diuron, DCPMU and atrazine) and, in a lesser extent, with various PAHs. The most vegetated area (HCV) was also the most contaminated. Indeed, for all contaminants (metals, PAHs and herbicides) but Ni and Cr, a clear decreasing gradient of contamination was observed from the HCV plot to the LCV plot; the ICV plot showing intermediate contamination levels (Figure 26).

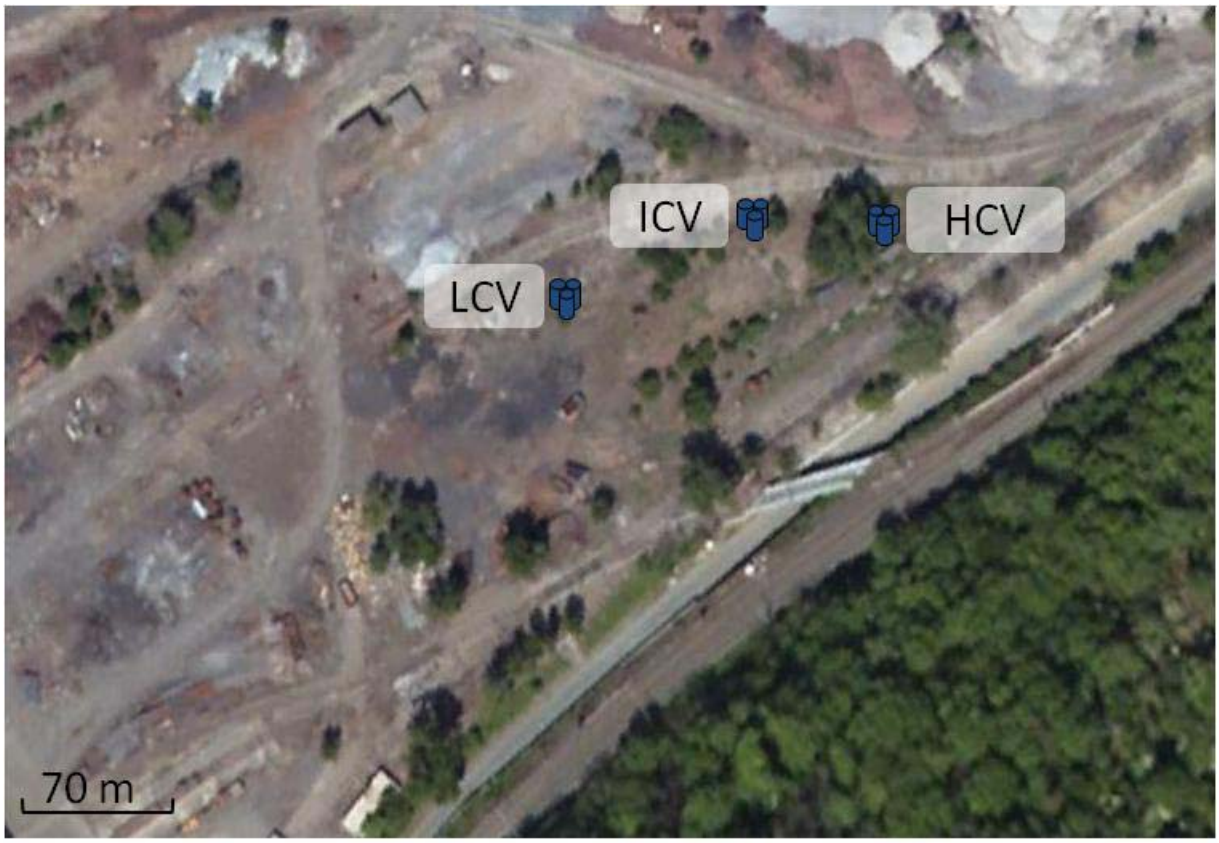


Figure 26: Localization of the microcosms on the plots of the SHSE site. LCV: low level of cover vegetation, ICV: intermediate level of cover vegetation, HCV: high level of cover vegetation. A blue cylinder represents a microcosm.

III.2.b. Plots description

Three plots were selected according to their level of plant cover with a gradient from “high level of cover of vegetation” plot (HCV) to “low level of cover of vegetation” plot (LCV) (Figure 27).

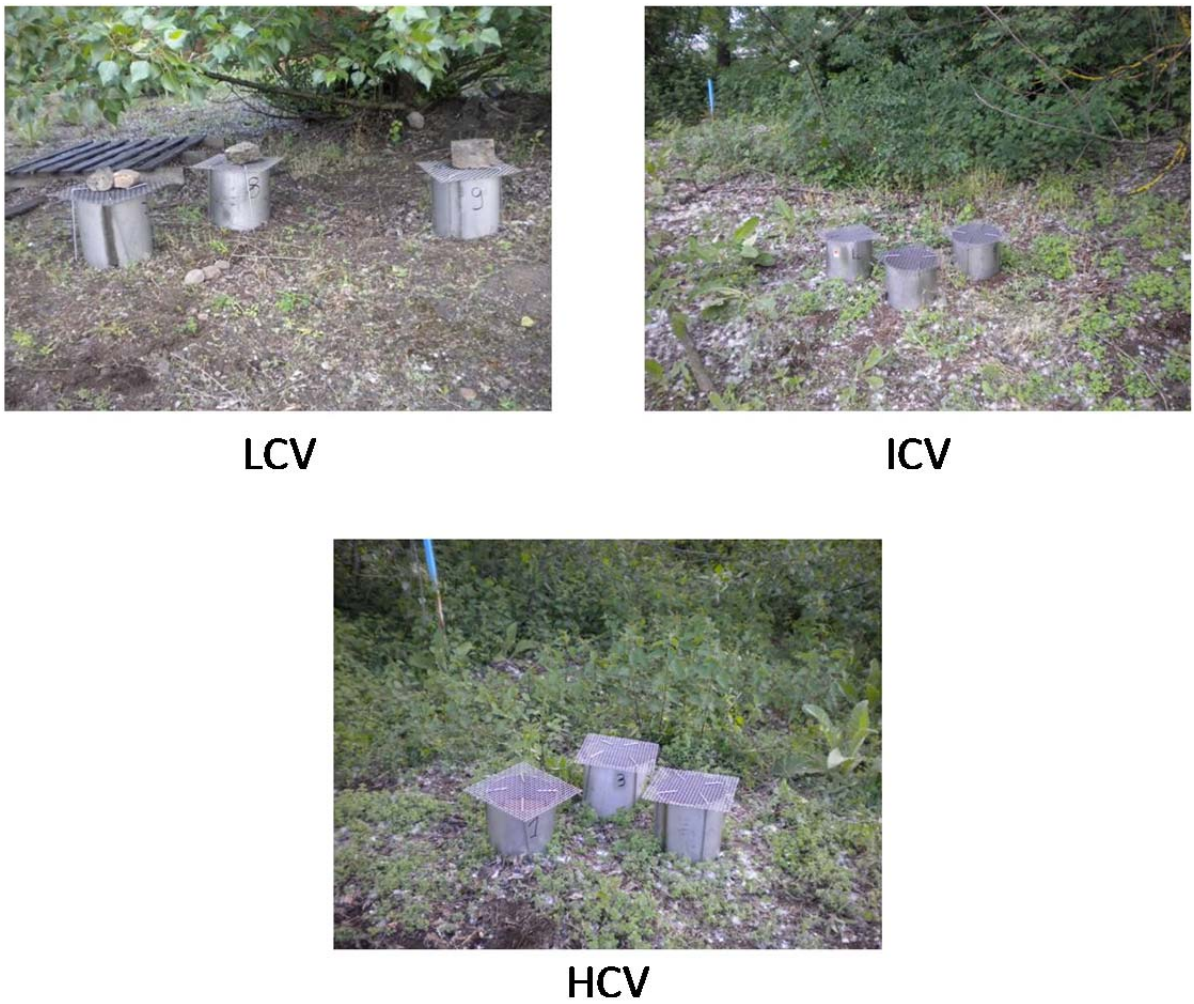


Figure 27: Snails exposure in microcosms on the plots of the SHSE site. LCV: low level of cover vegetation, ICV: intermediate level of cover vegetation, HCV: high level of cover vegetation.

III.3. Metaleurop

III.3.a. Site description

The Metaleurop site (a contaminated site, code MET) is located in northern France (50°29’ N, 2°59’E) and stretches around the former lead smelter of Noyelles-Godault, closed in 2003. It corresponds to areas polluted by atmospheric emissions from the smelter with a gradient of metal pollution (Cd, Pb, Zn) depending on the distance from the smelter. The climate is oceanic with a continental tendency, with a mean rainfall of 744 mm $year^{-1}$ and a mean annual temperature of 10.8°C.

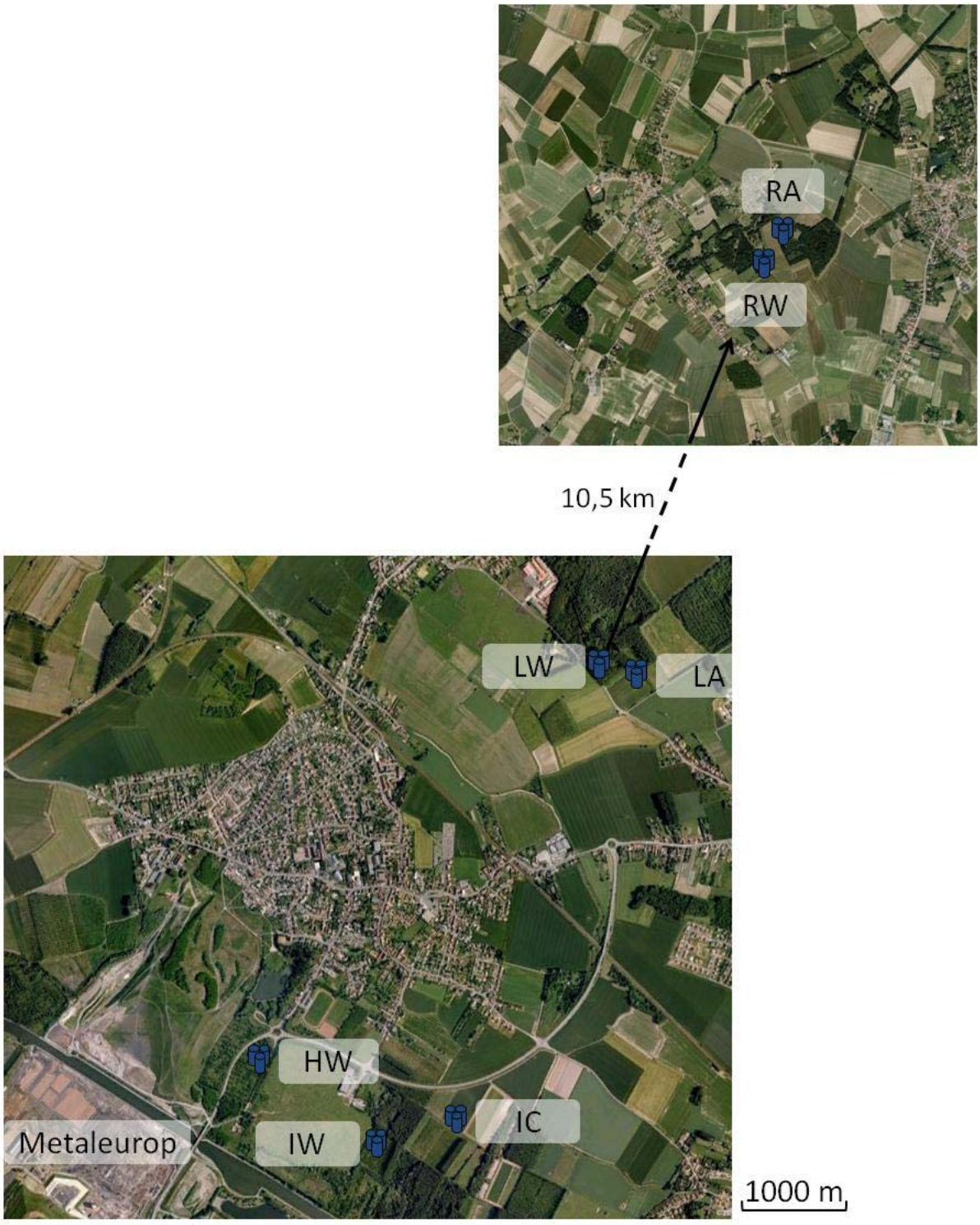


Figure 28: Localization of the microcosms on the plots of the Metaleurop site. R: reference, L: low metal contamination, I: intermediate metal contamination, H: high metal contamination, W: woodland, A: arable. A blue cylinder represents a microcosm.

III.3.b. Plots description

Four plots were selected according to a gradient of metal pollution in the vicinity (H, I and L for High, Intermediate and Low, respectively) or at 10 km (R for reference) of the former smelter (Figure 28),

and two soil occupancies were studied (woodland W and arable A) (Figure 29). Due to French law, any arable plot existed at 0.5 km from the smelter.

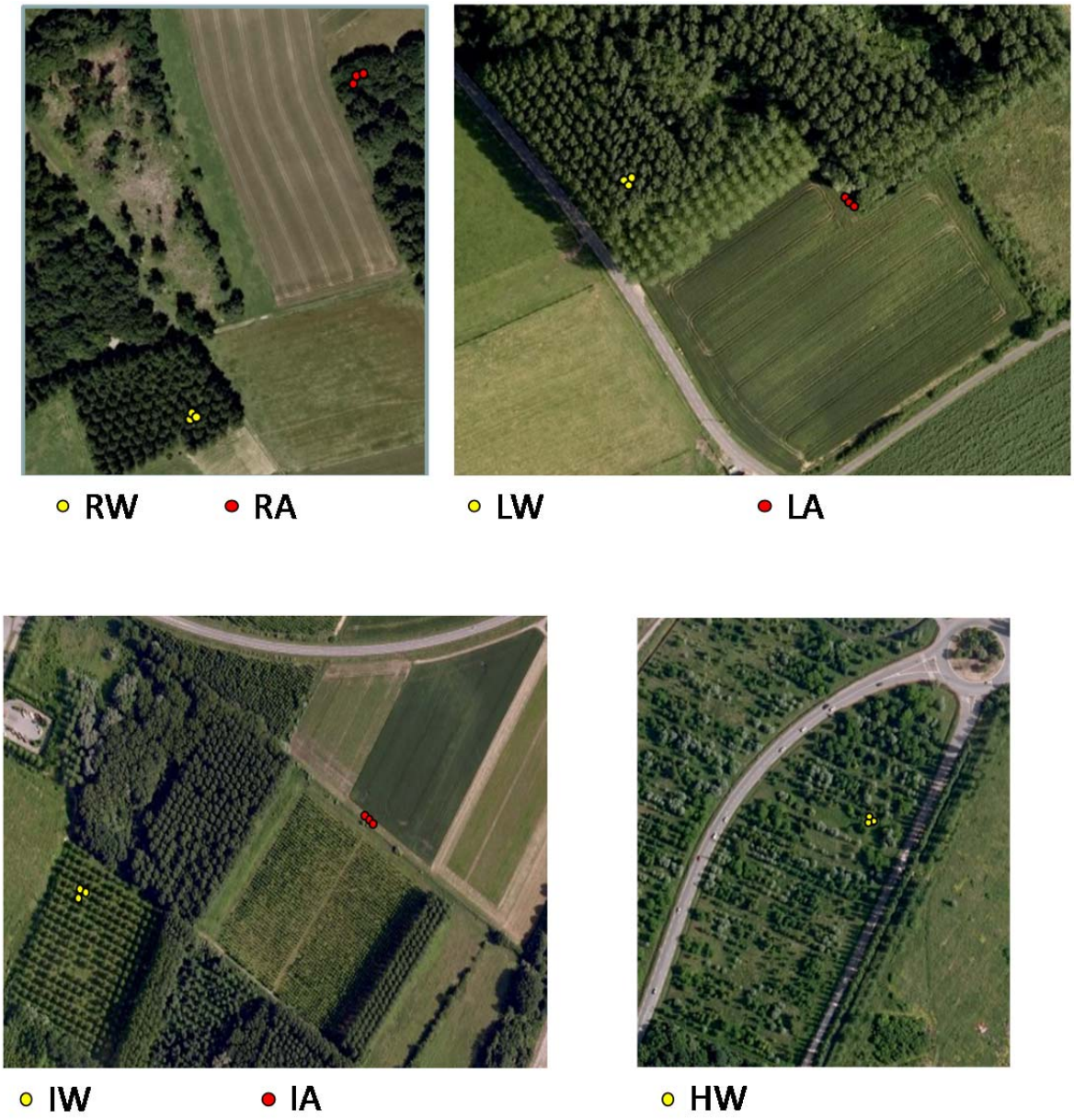


Figure 29: Snails exposure in microcosms on the plots of the Metaleurop site. R: reference, L: low metal contamination, I: intermediate metal contamination, H: high metal contamination, W: woodland, A: arable.

III.4. GISFI

III.4.a. Site description

The GISFI site is an industrial wasteland (a contaminated site, code GISFI), located at Homécourt (30 km west from Metz city, 49°21367N, 5°99608E), in north-eastern France. This experimental site of the French Scientific Interest Group – Industrial Wasteland (GISFI) (http://www.gisfi.fr) corresponds to a former industrial site with coking and metallurgical activities, closed in 1980. The climate is continental with a mean rainfall of 760 mm $year^{-1}$ and a mean temperature of 10°C. Soils were

mainly contaminated with organic pollutants such as PAHs and metallic pollutants, and exhibited high spatial heterogeneity.

Figure 30: Localization of the microcosms on the plots of the GISFI site. GHM: medium contamination, GHF: low contamination. A blue cylinder represents a microcosm.

III.4.b. Plot description

The two studied plots (Figure 30, GHF and GHM) presented the same contamination origin but differed by the levels of PAH and metal contamination (Table 6 and Figure 31). The plots presented the same PAH profile dominated by PAH with 3 and 4 cycles e.g. fluoranthène, phénanthrène and pyrène; metal contamination was dominated by Pb, Zn, Mo and Cd.

Figure 31: Snails exposure in microcosms on the plots of the GISFI site. GHM: medium contamination (left), GHF: low contamination (right).

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Chapitre 2 : Ranking field site management priorities according to their metal transfer to snails.

Abstract
Current soil quality evaluation does not include an assessment of metal bioavailability to organisms. However, sentinel soil-dwelling invertebrates can be used for such an assessment. This study aims to establish a procedure for ranking field sites (n = 9; 43 plots) based on the evaluation of the transfer of metals to the land snails used as indicators of metal zooavailability. Internal Concentrations of Reference (CIRef) of Cd, Pb, As, Cr, Cu and Zn were determined in *Cantareus aspersus* that were caged on unpolluted plots. Multivariate regressions were used to identify the soil characteristics that modulate metal zooavailability. CIRef allow for the identification of contaminated sites. CIRef have revealed unexpected metal transfer on some "unpolluted" sites and a lack of transfer on some contaminated sites, thus confirming the necessity for biological measures to evaluate metal mobility. Soil pH, organic carbon and iron oxides influenced metal bioavailability to snails. The Sum of Excess of Transfers (SET) index ranked the industrially impacted sites as the top priorities for management.
We recommend that the SET methodology be used for future environmental risk assessment. By highlighting real metal transfers and considering the numerous parameters influencing metal zooavailability, the snails watch provides information on environment quality.

Résumé
Les méthodes d'évaluations de la qualité des sols actuelles n'intègrent pas l'estimation de la biodisponibilité des métaux aux organismes. Pour palier à ce manque, les invertébrés inféodés au sol peuvent être utilisés en tant que sentinelles. Cette étude vise à établir une méthodologie pour hiérarchiser des sites (n=12 ; 43 modalités) basée sur l'évaluation des transferts de métaux aux escargots utilisés en tant qu'indicateur de la zoodisponibilité des métaux. Pour ce faire, les concentrations internes de référence (CIRef) du Cd, du Pb, de l'As, du Cr, du Cu et du Zn d'escargots *Cantareus aspersus* encagés sur des sites non contaminés ont été déterminées. Dans le but d'identifier les paramètres du sol influençant la zoodisponibilité des métaux pour l'escargot, des régressions multivariées ont été développées. Globalement les CIRef identifient les sites contaminés en métaux. Elles révèlent également des transferts de métaux inattendus sur des sites à priori « non contaminés » ainsi qu'une absence de transfert sur un site contaminé soulignant la nécessité des mesures biologiques pour évaluer la mobilité et la zoodisponibilité des métaux. Le pH, le carbone organique ainsi que les oxydes de fer ont été identifiés comme modulant la zoodisponibilité des métaux pour l'escargot. L'indice Somme des Excès de Transfert (SET) permet de classer les sites industriels en tête de liste des priorités de gestion. Nous recommandons l'utilisation de l'indice SET pour l'évaluation des risques environnementaux futurs. En effet, en soulignant le transfert du métal réel et en tenant compte des nombreux paramètres qui influencent la zoodisponibilité des métaux, cet outil montre que les escargots fournissent des informations sur la qualité de l'environnement.

I. Introduction

Current risk assessment procedures assume that the total amount of metal contaminants in soil is available for uptake by organisms, including humans. For instance, risk management in France (IEM, 2007) focuses first on soil use (e.g., gardens and parking) and secondly on the estimated exposure, based on the total concentration of metal in the soil. However, it is now recognized that soil characteristics such as pH or organic matter content (OM) influence the environmental and toxicological bioavailability of metals (Lanno et al., 2004; van Gestel, 2008) to organisms such as earthworms (Nahmani et al., 2007) or snails (Pauget et al., 2011). To prevent misinterpretation, soil risk assessment must consider metal transfer, based on the total concentration, as modulated by soil characteristics and for selected ecological receptors (Allen, 2002; Luoma and Rainbow, 2005). Toward this aim, using biological data, such as the internal concentration of contaminants in soil invertebrates, is suitable because it considers both physicochemical and biological processes that modulate metal transfer from the soil to fauna. Living at the interface between soil, plants and air, snails provide information on both the retention and habitat functions of soil (ISO 17402, 2008). Moreover, the garden snail, as several land snail species, is involved in transfer of MTEs in food webs, because of its abilities to accumulate great amounts of metals in soft tissues and its position in trophic webs (detrivorous and herbivorous species, being dietary item of numerous invertebrate and vertebrate predators) (Laskowski and Hopkin, 1996; Gomot-de Vaufleury and Pihan, 2002; Allen, 2004). Snails are efficient soil quality bioindicators for *in situ* soil surveys using passive biomonitoring of wild organisms (Berger and Dallinger, 1993; Mourier et al., 2011). Active biomonitoring can also be performed, such as an assessment of metal transfer by analyzing the internal concentrations of chemicals in *Cantareus aspersus* with a known biological past, with the snails being caged for given durations on the studied sites (Fritsch et al., 2011). This approach first provides information regarding the bioavailability to snails of the metals in soils, and second provides information on the amount of contaminants in the soil that could be transferred through terrestrial food chains involving snails and their consumers (Vermeulen et al., 2010; Soto et al., 2011), including humans in some countries. Although the land snail *C. aspersus* has already been used in active biomonitoring for many metals on various field sites (Gomot de Vaufleury and Pihan, 2000; Regoli et al., 2006), no large-scale attempt to create a framework usable for risk assessment has been performed. Moreover, while the influence of soil properties on metal bioavailability to snails has been investigated under laboratory conditions (Pauget et al., 2011), it has never been quantified *in situ*.

To improve soil quality assessment and risk management of contaminated sites by using relevant soil quality bioindicators, a French program has been run for 3 years on 12 field sites (Bioindicators program, (Bispo et al., 2009)). Within this framework, the present study aims to determine the metal concentrations of the land snail *C. aspersus* caged in unpolluted sites; the internal concentrations of reference (CIRef) were determined for cadmium (Cd), lead (Pb), arsenic (As), chromium (Cr), copper (Cu) and zinc (Zn). The CIRef will then be used to distinguish sites that present metal transfer to snails. The second objective is to determine how metal accumulation in snails is influenced by soil properties using mono- and multivariate regressions. The third objective is to develop a method to evaluate the transfer of metals, using the CIRef to synthesize data of sites contaminated with multiple metals and to determine handling priorities, based on the total metal concentration in soil and biological assessments of metal bioavailability.

II. Materials and Methods

II.1. Animals

Juvenile land snails (*C. aspersus*) were reared under controlled conditions, as described by Gomot-de Vaufleury (Gomot-de Vaufleury, 2000), and fed with uncontaminated (0.353 mg kg^{-1} Cd DW (dry weight), 0.696 mg kg^{-1} Pb DW, 1.11 mg kg^{-1} As DW, 14.5 mg kg^{-1} Cr DW, 10.4 mg kg^{-1} Cu DW, 182 mg kg^{-1} Zn DW) commercial snail meal (Helixal®, Berthon S.A., France). The individuals used for the exposure were subadults, 7 to 9 weeks old and weighing 5.0±0.6 g (n=1230). At the beginning of the exposure, the metal concentrations in the snails' viscera were 0.73±0.10 mg kg^{-1} Cd DW, 0.59±0. 26 mg kg^{-1} Pb DW, 0.33±0.11 kg^{-1} for As DW, 2.19±0.48 mg kg^{-1} Cr DW, 139±40.1 mg kg^{-1} Cu DW and 881±182 mg kg^{-1} Zn DW (mean±SD, n=10).

II.2. Soils

Nine sites were selected throughout France, falling into three categories (cultivated sites, forests and contaminated sites) and five land uses (forest, arable, pasture, woodland and wasteland) (Figure 32, http://ecobiosoil.univ-rennes1.fr/ADEME-Bioindicateur). These sites represent a large range of metal contamination and soil parameters, as summarized in Table 7. The sites of Auzon, Metaleurop, the slag heap of Saint-Etienne (SHSE) and GISFI are sites impacted by industrial activities, which generally have high soil metal pollution (Table 7). The forest sites, part of the RENECOFOR network (http://www.onf.fr/renecofor), have low metal concentrations in their soils (Table 7). The sites of Qualiagro (Houot et al., 2002), BioREco, Yvetot and Thil are cultivated sites and have slight metal concentrations in soils (Table 7). Some of the sites were studied in 2009 (Qualiagro, Metaleurop, F08, F57, GIS-FI) and were previously described in Pérès et al (Pérès et al., 2011). A brief description of the sites studied in 2010 is given here:

The Auzon site (45°23'12N, 03°21'32 E) is an industrial waste site with trace element contamination, such as arsenic. Six plots (4 contaminated "Co", 2 controls "Ct") were selected according to a metal pollution gradient (from 62 to 3600 mg.kg^{-1} As) and vegetation cover (woodland "W", woodland on hydromorphic soil "WW", woodland hedge "WH" and wasteland "Wa").

The SHSE site is a metallurgical landfill near Saint-Etienne (45°43'N, 4°39'E), with high metal contamination combined with herbicide contamination. Three plots were selected according to their level of plant cover, with a gradient from the "high level of cover of vegetation" plot (HCV) to the "low level of cover of vegetation" plot (LCV).

The BioREco site is an experimental site from INRA (Institut National de la Recherche Agronomique) (44°97'N, 4°92 E) created in 2005 (Simon et al., 2011). Six plots were selected, combining three different protection strategies (conventional supervised (CV), low input (LI) and organic farming (OG)) and two apple cultivars with different susceptibility to disease ("Ariane" susceptible to scab (A) and "Golden Delicious" resistant to scab (GD)).

The Yvetot site is an agricultural site (49°36'N, 0°44'E) on which different crop/pasture management practices are tested (Plassart et al., 2008). Six plots were selected: one arable cropping (GC), two rotation pastures (RP1, RP2), which correspond to restored pastures after 5-6 yr of cropping period, two temporary pastures (TP1, TP2), which correspond to different arable-grassland rotations, and one permanent pasture (PP).

The Thil site is an agricultural site (45°49' N, 5°22 E) managed for organic farming on which different tillage managements have been compared since 2005 (Vian et al., 2009). Four plots were selected:

(i) Traditional Moldboard Plowing (MP); (ii) Shallow Moldboard Plowing (SMP); (iii) Reduced Tillage (RT) with tined tools; and (iv) Superficial Tillage (ST).

The F63 forest site is a Common Spruce forest (45°75'N, 2°96'E), and the F76 site is a Scots Pine forest (49°45'N, 0°74 E).

Soil analyses were performed by the *Soil laboratory of Arras* (France), which benefits from the COFRAC (French accreditation committee) accreditation n°1-1380 (available at www.cofrac.fr) for its analytical quality for metal measurements in soils. More details on sampling strategy and analysis are presented in Pérès et al (Pérès et al., 2011).

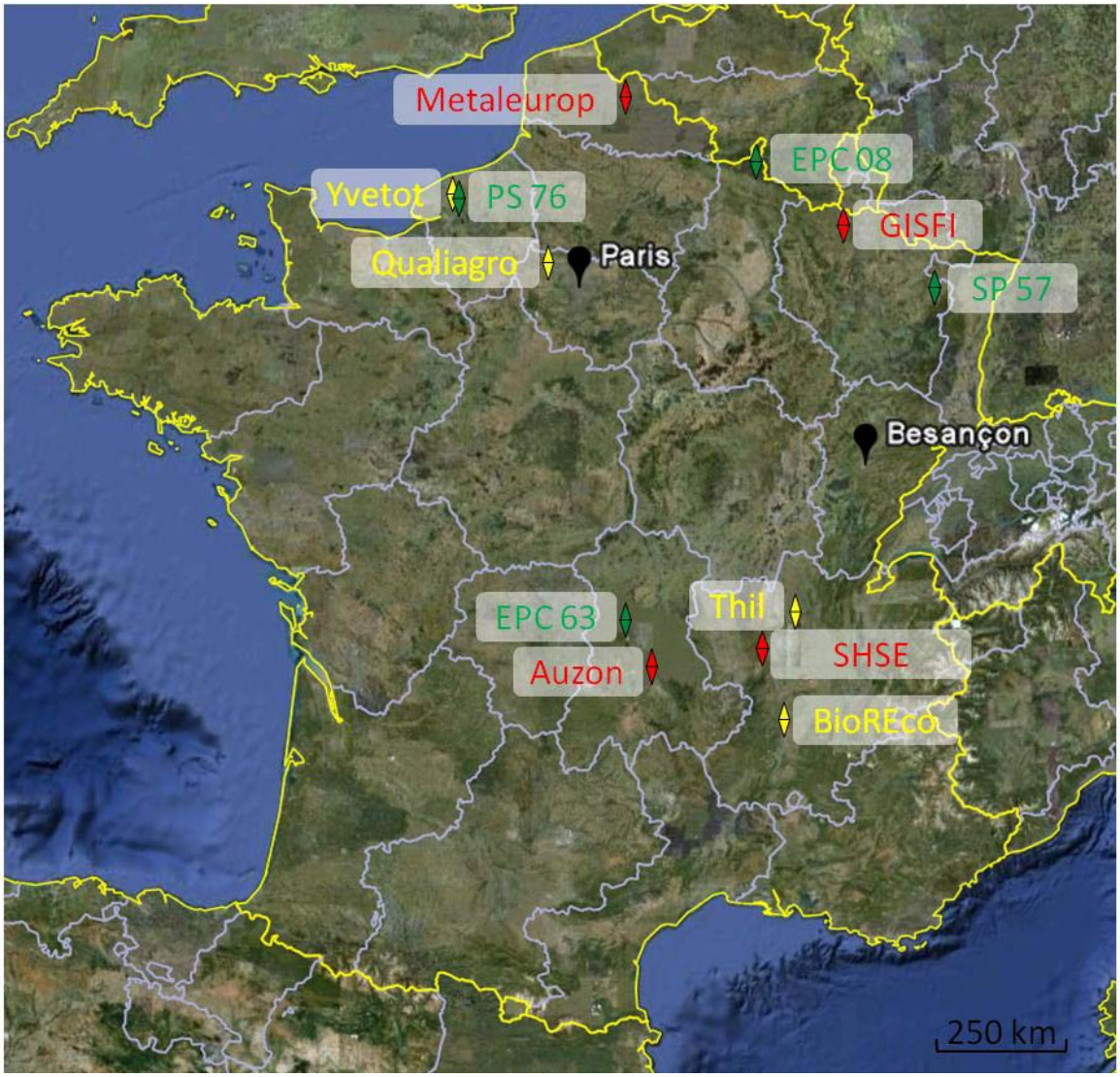

Figure 32: Sites localization of the Bioindicators program. Red: industrial sites, yellow: farm sites, green: forest sites (RENECOFOR). http://ecobiosoil.univ-rennes1.fr/ADEME-Bioindicateur/

Table 7: Medians of physicochemical characteristics of the 12 Bioindicator program sites studied and of concentrations of metals in snail viscera exposed in microcosms for 28 days. Bold values identify a pollution of the soil by the trace element.

Site	Plot	Soil														Snail viscera concentration (mg_{metal} kg_{sn}^{-1} DW)					
		pH	Silts ($g\ kg^{-1}$)	Sands ($g\ kg^{-1}$)	OM ($g\ kg^{-1}$)	Clay ($g\ kg^{-1}$)	CEC ($cmol\ kg^{-1}$)	C_{org} ($g\ kg^{-1}$)	$[Al]_{ox}$ ($cmol\ kg^{-1}$)	$[Fe]_{ox}$ ($cmol\ kg^{-1}$)	$[Cd]_{tot}$ ($mg\ kg^{-1}$)	$[Pb]_{tot}$ ($mg\ kg^{-1}$)	$[As]_{tot}$ ($mg\ kg^{-1}$)	$[Cr]_{tot}$ ($mg\ kg^{-1}$)	$[Cu]_{tot}$ ($mg\ kg^{-1}$)	$[Zn]_{tot}$ ($mg\ kg^{-1}$)	$[Cd]_{snail}$	$[Pb]_{snail}$	$[As]_{snail}$	$[Cr]_{snail}$	$[Cu]_{snail}$	$[Zn]_{snail}$
GISFI	GHF	8.30	225	611	209	143	15.0	121	0.067	0.019	**0.499**	**165**	**32.8**	57.1	**30.3**	**408**	0.167	9.87	0.114	1.34	94.9	821
	GHM	8.33	172	736	363	94.0	11.0	210	0.026	0.024	**1.23**	**309**	**58.5**	**171.50**	**45.3**	**323**	0.176	13.2	0.179	0.697	143	762
Metaleurop	HW	7.99	601	101	83.5	298	29.3	48.3	0.038	0.014	**34.4**	**2485**	**39.3**	48.6	**68.4**	**1885**	10.9	112	0.241	0.03	168	1181
	IW	8.19	609	96.0	46.0	294	24.3	26.6	0.040	0.020	**13.3**	**731**	**30.9**	50.3	**27.6**	**745**	6.03	34.8	0.196	0.213	136	993
	IA	8.21	474	211	45.1	318	29.3	26.1	0.054	0.014	**8.54**	**482**	18.1	61.5	**28.7**	**509**	1.76	14.3	0.237	0.581	141	916
	LW	6.45	283	517	55.0	203	17.2	31.8	0.059	0.015	**5.44**	**319**	9.42	52.0	19.4	**332**	9.94	48.7	0.250	0.588	153	1304
	LA	7.90	366	452	29.3	181	13.2	16.9	0.078	0.020	**3.12**	**143**	8.67	49.4	15.1	**226**	5.73	61.4	0.301	0.313	161	1599
	RW	6.55	525	313	34.9	163	12.2	20.2	0.053	0.011	**1.09**	48.8	7.11	41.5	12.3	**102**	8.24	13.8	0.369	0.03	136	1651
	RA	6.96	531	290	25.5	181	13.0	14.7	0.048	0.010	**0.968**	41.7	8.60	45.1	14.2	92.0	2.60	9.95	0.383	0.03	106	887
Auzon	CoWW	5.41	427	346	103	238	19.1	59.6	0.425	0.015	**9.97**	**4575**	**3285**	76.5	**159**	**225**	2.28	7.48	1.975	0.694	149	903
	CoW	5.73	279	574	70.6	148	13.0	40.8	0.176	0.016	**0.722**	**104**	**339**	57.9	**22.9**	**148**	2.01	2.85	0.571	0.696	122	1039
	CoWH	5.23	167	757	76.5	89.5	11.0	44.2	0.410	0.017	**1.32**	**282**	**661**	51.8	**38.6**	**140**	1.83	4.01	1.33	0.589	122	866
	CoWa	5.81	167	713	60.3	98.5	9.46	34.9	0.111	0.009	**6.74**	**1834**	**1087**	52.3	**140**	**173**	2.75	14.2	1.22	0.478	120	1149
	CtW	6.22	299	556	70.7	138	14.9	40.9	0.069	0.012	**0.612**	60.1	**123**	67.3	**27.7**	**138**	2.32	4.30	2.24	0.630	130	746
	CtWH	5.10	225	628	45.2	148	8.90	26.2	0.814	0.016	0.143	28.1	**62.5**	56.8	**22.8**	88.1	1.30	2.35	0.255	0.804	127	1026
SHSE	HCV	8.10	142	803	81.1	48.5	10.5	46.9	0.081	0.030	**21.0**	**2525**	**73.3**	**982**	**1555**	**2830**	2.84	14.2	0.243	2.34	92.2	1466
	ICV	8.74	131	820	52.5	44.5	6.60	30.4	0.065	0.026	**9.37**	**1616**	**54.8**	**1158**	**570**	**2180**	5.23	42.1	0.263	1.54	145	1683
	LCV	8.57	77	886	25.6	33.5	3.47	14.8	0.070	0.031	**1.99**	**513**	**30.4**	**4345**	**525**	**577**	2.56	26.2	0.566	2.60	121	1913
Qualiagro	Ct	6.84	783	66.5	17.7	156	8.42	10.2	0.072	0.015	0.206	22.1	7.36	47.7	12.0	50.1	1.99	13.6	0.067	1.40	60.4	608
	FYM	7.46	777	67.0	24.4	156	9.74	14.1	0.069	0.014	0.226	26.7	7.22	47.4	15.8	58.4	1.47	8.58	0.067	1.09	137	777
	BIOW	7.81	771	68.5	25.2	161	10.50	14.6	0.080	0.016	0.230	25.6	7.83	45.0	14.0	57.7	2.36	10.8	0.114	1.36	105	712
	GWS	7.00	780	72.0	26.3	151	9.60	15.2	0.078	0.016	0.222	24.2	6.77	48.6	16.4	59.0	1.89	5.09	0.067	0.97	114	772
	MSW	7.62	774	71.0	21.5	155	9.64	12.4	0.070	0.014	0.231	28.3	7.26	47.0	15.2	58.9	1.42	3.77	0.067	1.05	95.9	721
Yvetot	RP1	5.58	665	196	19.2	140	5.43	11.1	0.208	0.008	0.236	21.0	6.93	66.3	10.3	42.7	0.972	4.07	0.001	0.533	69.0	813
	RP2	6.03	665	210	24.8	128	6.97	14.4	0.066	0.009	0.243	30.6	6.76	49.0	14.4	47.0	1.07	3.37	0.020	0.753	74.8	532
	PT2	6.25	655	204	23.9	142	7.74	13.8	0.058	0.010	0.217	27.9	8.57	51.3	14.8	50.7	1.78	7.14	0.077	1.11	68.0	927
	PT1	5.50	652	202	32.6	149	7.25	18.9	0.172	0.007	0.205	21.4	8.52	51.2	13.7	48.4	1.01	2.22	0.054	0.883	80.7	1013
	PP	5.48	634	203	44.6	163	8.06	25.8	0.255	0.008	0.198	26.3	9.48	53.8	13.4	51.5	1.07	3.98	0.001	0.789	84.2	823
	GC	6.42	670	197	18.6	132	7.02	10.8	0.050	0.009	0.199	19.0	8.14	49.2	10.5	45.4	1.75	4.85	0.001	1.43	45.6	788
BioREco	OG_A	7.13	261	533	21.4	204	9.39	12.3	0.110	0.023	0.147	23.7	12.3	53.6	24.4	47.4	1.17	4.36	0.001	1.12	151	1045
	OG_GD	7.10	292	556	18.4	158	6.93	10.6	0.106	0.021	0.164	22.4	9.36	44.7	**26.3**	41.8	0.960	3.08	0.001	0.700	188	845
	LI_A	7.35	286	528	24.9	186	9.39	14.4	0.139	0.027	0.148	26.5	10.6	48.2	19.5	46.6	1.52	4.87	0.001	0.901	144	1104
	LI_GD	7.28	293	520	22.9	184	8.56	13.3	0.094	0.018	0.165	29.3	11.4	48.9	**32.2**	49.3	1.26	7.34	0.001	0.676	136	821
	CV_A	6.89	297	540	23.9	162	6.91	13.8	0.082	0.016	0.182	31.1	10.4	46.4	19.8	42.2	1.11	4.80	0.001	0.722	95.8	1030
	CV_GD	7.36	296	545	25.1	164	8.61	14.5	0.095	0.018	0.178	26.2	10.3	47.6	**31.9**	42.9	1.21	6.96	0.001	1.19	125	1034
Thil	ST	8.13	235	617	27.7	143	9.78	16.0	0.036	0.007	0.212	14.6	6.55	43.3	8.22	41.9	2.26	5.91	0.001	0.03	101	1021
	SMP	8.23	224	641	23.5	130	8.91	13.6	0.061	0.012	0.227	14.7	6.53	44.8	8.91	42.3	1.20	3.57	0.001	0.03	122	970
	MP	8.28	181	720	19.8	100	6.59	11.4	0.050	0.010	0.189	12.8	5.67	36.5	7.57	35.3	1.17	6.83	0.001	0.03	105	1058
	RT	8.20	212	673	21.9	124	8.39	12.6	0.056	0.010	0.209	14.3	6.56	43.4	8.92	40.1	1.32	7.65	0.001	0.03	105	965
RENECOFOR	F08	4.05	630	106	151	262	10.4	87.4	7.00	0.126	0.235	**78.9**	**25.4**	57.9	17.1	34.0	1.26	50.6	0.067	0.670	67.0	711
	F57	4.13	73.0	886	22.5	44.5	2.14	13.0	1.43	0.027	0.025	21.1	3.83	6.55	1.73	8.62	1.88	8.29	0.067	1.01	78.0	826
	F76	3.77	354	566	180	78	7.10	104	1.87	0.059	0.063	21.9	2.91	17.7	3.48	13.5	1.27	2.55	0.128	1.25	84.7	1403
	F63	4.87	411	326	269	257	9.42	156	5.64	0.046	0.414	49.7	12.8	55.0	14.1	**113**	1.15	3.30	0.261	1.27	104	1507

II.3. Exposure modality

Microcosms (25 x 25 cm stainless steel cylinders, similar to those used by Fritsch et al. (Fritsch et al., 2011)) caged the snails for the exposure time (28 days) at the different sites. Snails were exposed to the soil and vegetation of each plot under natural climatic conditions.

Fifteen snails were exposed in each microcosm. On forest and cultivated sites, one microcosm per plot was used and 6 snails were sampled for metal analysis after 28 days. On sites contaminated by industrial activities, 3 microcosms per plot were used to prevent soil heterogeneity contamination, and two snails per microcosm (=6 snails) were sampled for metal analysis after 28 days.

II.4. Metal analysis in snails

Sampled snails were fasted for 48 h (the feces were removed after 24 h) and then weighed and sacrificed by freezing at -80°C. After thawing, the whole soft body was removed from the shell and the foot was separated from the viscera. The viscera were studied because they are the main site of metal accumulation in snails (Hopkin, 1989). The viscera were oven-dried at 60°C until they reached a constant weight (~ 0.2/0.3 g DW), digested in nitric acid (65% HNO_3, Carlo-Erba analytical quality) as previously described (Pauget et al., 2011) and analyzed by ICP-MS. The validity of the analytical methods was checked by analyzing standard biological reference material (TORT-2, lobster hepatopancreas; National Research Council of Canada-Institute for National Measurement Standard, Ottawa, ON, Canada).

II.5. Assessment and use of the snails' CIRef

A two-step approach was developed to establish the metal concentrations in snails caged in unpolluted sites (i.e., the CIRef).

First step: Determination of the unpolluted plots.

The first step was to determine which soils would be considered as "unpolluted". Because of the lack of a legal reference in France, three concentrations were retained as threshold values of soil pollution for each metal: (1) the highest limit of the range of "normal concentrations in French soil" proposed for the metals studied in the ASPITET program (Baize, 2000); (2) the upper whisker of the soil concentrations of the RMQS program (Villanneau et al., 2008); and (3) the BDETM program (Baize et al., 2007). These threshold values are presented in Table 8. The plots studied here were classified as "unpolluted" when the mean of their soil concentration was lower than the threshold value. Thus, the use of three threshold values led to three different pools of unpolluted plots being defined, except for As, which only had one available threshold value (ASPITET program).

Second step: CIRef calculation

For each of the three pools of unpolluted plots, a theoretical CIRef was calculated for each metal as follows. First, the quartile distributions of the concentrations in snails exposed on each unpolluted plot were determined, and the outliers, corresponding to the concentrations that were higher than the upper whisker (75th percentile + 1.5 * Interquartile range (Tukey, 1977)), were removed. Then, the remaining data were pooled and the upper whisker was recalculated. Finally, three theoretical CIRef were obtained according to the soil threshold values used (Table 8) and the lowest one was chosen as the final CIRef.

Use of CIRef

To identify whether a site or a plot within a site presents a metal transfer to snails, the median of the snail's viscera concentration is compared to the CIRef. If the median concentration in

the snail exposed on the plot under investigation was higher than the CIRef, then the conclusion is that the soil presents an abnormal metal transfer to snail.

II.6. Soil parameters influence on metal accumulation in snails

To determine the influence of soil parameters on metal accumulation, a two-step approach was used. First, the influence of total soil concentration on metal accumulation was estimated using monovariate regression. Then, multivariate regressions of the internal concentration after 28 days of exposure (C_{28days}) against metal soil concentration coupled to soil parameters were performed (Pauget et al., 2011) to estimate the soil parameter's influence (Eq. 1):

(1) $$Y = x * A + y * B + ,..., z$$

where Y = log($C_{28days\text{-}viscera}$+1), x, y, … represent the coefficients and A, B, … represent the soil characteristics (pH and log+1 of total soil metal concentration, CEC, OM and clay content, exchangeable aluminum and iron oxides (evaluated using cobaltihexamine extractant), organic carbon, sand and silt). The best model was chosen using AICc (Burnham and Anderson, 2004).

II.7. Evaluation of the transfer of metal: the Sum of the Excess of Transfer (SET) methodology

First, an accumulation quotient (AQ) was calculated for each metal using equation (2):

(2) $$AQ_{metal} = \frac{C_{28days}}{CIRef}$$

where AQ_{metal} is the accumulation quotient of the metal (representing the metal transfer), C_{28days} is the concentration of the metal in the snails' viscera after 28 days of exposure (mg.kg^{-1}) and CIRef is the internal concentration of reference of the metal (mg.kg^{-1}).

An abnormal metal transfer to snails is characterized by an AQ > 1, and higher AQ values indicate greater transfer.

In the case of a normal metal transfer, the calculated AQ is ≤1 and will always be adjusted to 1 in the next step of calculation.

The global metal transfer is calculated from the sum of the excess of transfer at each site (SET_{site}) and each plot (SET_{plot}) according to (3 and 4):

(3) $$SET_{plot} = \sum (AQ_{metal} - 1)$$

(4) $$SET_{site} = \frac{\sum SET_{plot}}{n_{plot}}$$

where SET represents the global excess of metal transfer, calculated using the excess of each metal transfer (AQ_{metal}-1, where 1 represents normal transfer), and n_{plot} is the number of plots used. SET_{site} is equal to SET_{plot} when the site has only one plot.

III. Results

III.1. Snails mortality

During the exposure, only 11% of exposed snails have died. This mortality is homogenous between the plots and allows enough remaining snails for the analysis to be obtained.

III.2. Establishment of the CIRef values

CIRef for Cd, Pb, As, Cr, Cu and Zn, based on metal concentrations in snails exposed to unpolluted plots (not bold values in Table 7), are presented in Table 8 and represent a benchmark. The retained CIRef (in bold in Table 8) are those obtained using the soil threshold values of the ASPITET program (for Cd, As, Cr, Cu and Zn) and the RMQS program (for Pb). Except for Pb, the selected CIRef corresponds to the most restrictive threshold soil values.

Table 8 : Soil threshold values (mg metal.kg^{-1} dry soil) used to determine polluted/unpolluted soils and corresponding CIRef (mg_{metal} kg_{sn}^{-1} DW) for metals.

Metal	Item	RMQS	ASPITET	BDETM
Cd	Soil threshold value	0.67	**0.45**	0.8
	CIRef	*2.48*	***2.27***	*2.48*
Pb	Soil threshold value	**62.3**	50	52.4
	CIRef	***12.9***	*13.1*	*13.1*
As	Soil threshold value		**25**	
	CIRef		***0.307***	
Cr	Soil threshold value	116	**90**	91.8
	CIRef	*2.01*	***2.01***	*2.01*
Cu	Soil threshold value	42.7	**20**	33.3
	CIRef	*216*	***185***	*216*
Zn	Soil threshold value	161	**100**	122
	CIRef	*1581*	***1490***	*1581*

III.3. CIRef: a tool identifying abnormal metal transfer

The soil pollution characterization using the threshold values and the identification of metal transfer to snails is presented in Table 9 and Figure 33.

Most of the unpolluted sites, as identified by the soil threshold values, did not show any transfer of metals to snails. However, 3.85%, 7.41%, and 6.90% of all plots showed a metal transfer for Cd (plot BIOW), Pb (plots RW and Ct), and As (plots RA, RW), respectively (i.e., an AQ > 1), although the soil concentrations of metal were lower than the threshold values. For the three other metals, no transfer to snails exposed on the unpolluted plots was observed.

Metal transfer to snails was identified in 70.6%, 68.8% and 42.9% of the polluted plots for Cd, Pb and As, respectively. Although the soils in the plots at the GISFI site (GHF and GHM) were identified as being polluted by metals, the comparison between the internal metal concentration and the CIRef shows a normal level of metal transfer, except for Pb in the GHM plot. A lack of metal transfer in the polluted plots was also observed at the Auzon site, where only three (for Cd) and one (for Pb) of the five polluted plots exhibited abnormal levels of metal transfer. At the Metaleurop site, As transfers were only observed in unpolluted plots (RA and RW), with no transfers occurring in the polluted plots (plots IW and HW).

For Cu, Zn, and Cr, only 5.6%, 29.4% and 50.0%, respectively, of the polluted plots exhibited metal transfer to snails. For Cu, the only transfer was observed in the OG_GD plot (cultivated site of BioREco). The few Zn transfers identified in the polluted plots do not correspond to the soil pollution gradient. Indeed, the plots where a Zn transfer occurred were not the most polluted ones (LA, RW, ICV, LCV and F63). A transfer of Cr was observed in only two of the 3 polluted plots at the SHSE site.

III.4. Influence of soil properties on metal accumulation

Total metal concentrations in the soil explain a large part of the metal accumulation in snails for Cd, Pb and As (r^2_{adj} = 0.43, 0.37 and 0.67, respectively, Table 10 and Annexe 2). For Cr, Cu and Zn, the

influence of total soil concentration was found to be lower (R^2_{adj} = 0.20, 0.09 and 0.17, respectively, Table 10 and Annexe 2). Adding the soil characteristics improves the assessment of metal accumulation in snails' viscera for all the metals except As. The main parameters that modulate metal accumulation are organic carbon content (for Cd, Pb, Cr and Zn) and aluminum and iron oxides (for Pb, Cu and Zn). The influence of soil pH (for Pb), of CEC (for Cr), of sands content (for Cr and Zn), of clay and silts content (for Cu) and of OM content (for Zn) was also noted (Table 10).

Table 9: Classification of soils from the 12 sites as polluted or not and metal transfer to snails.

Site	Plot	Cd		Pb		As		Cr		Cu		Zn	
		Soil	metal Transfer	Soil	metal Transfer	Soil	metal Transfer	Soil	metal Transfer	Soil	metal Transfer	Soil	metal Transfer
GISFI	GHF	**Poll.**	No	**Poll.**	No	**Poll.**	No	UnP	No	**Poll.**	No	**Poll.**	No
	GHM	**Poll.**	No	**Poll.**	**Trans.**	**Poll.**	No	**Poll.**	No	**Poll.**	No	**Poll.**	No
Metaleurop	HW	**Poll.**	**Trans.**	**Poll.**	**Trans.**	**Poll.**	No	UnP	No	**Poll.**	No	**Poll.**	No
	IW	**Poll.**	**Trans.**	**Poll.**	**Trans.**	**Poll.**	No	UnP	No	**Poll.**	No	**Poll.**	No
	IA	**Poll.**	No	**Poll.**	**Trans.**	UnP	No	UnP	No	**Poll.**	No	**Poll.**	No
	LW	**Poll.**	**Trans.**	**Poll.**	**Trans.**	UnP	No	UnP	No	UnP	No	**Poll.**	No
	LA	**Poll.**	**Trans.**	**Poll.**	**Trans.**	UnP	No	UnP	No	UnP	No	**Poll.**	**Trans.**
	RW	**Poll.**	**Trans.**	UnP	**Trans.**	UnP	**Trans.**	UnP	No	UnP	No	**Poll.**	**Trans.**
	RA	**Poll.**	**Trans.**	UnP	No	UnP	**Trans.**	UnP	No	UnP	No	UnP	No
Auzon	CoWW	**Poll.**	**Trans.**	**Poll.**	No	**Poll.**	**Trans.**	UnP	No	**Poll.**	No	**Poll.**	No
	CoW	**Poll.**	No	**Poll.**	No	**Poll.**	**Trans.**	UnP	No	**Poll.**	No	**Poll.**	No
	CoWH	**Poll.**	No	**Poll.**	No	**Poll.**	**Trans.**	UnP	No	**Poll.**	No	**Poll.**	No
	CoWa	**Poll.**	**Trans.**	**Poll.**	**Trans.**	**Poll.**	**Trans.**	UnP	No	**Poll.**	No	**Poll.**	No
	CtW	**Poll.**	**Trans.**	**Poll.**	No	**Poll.**	**Trans.**	UnP	No	**Poll.**	No	**Poll.**	No
	CtWH	UnP	No	UnP	No	**Poll.**	No	UnP	No	**Poll.**	No	UnP	No
SHSE	HCV	**Poll.**	**Trans.**	**Poll.**	**Trans.**	**Poll.**	No	**Poll.**	**Trans.**	**Poll.**	No	**Poll.**	No
	ICV	**Poll.**	**Trans.**	**Poll.**	**Trans.**	**Poll.**	No	**Poll.**	No	**Poll.**	No	**Poll.**	**Trans.**
	LCV	**Poll.**	**Trans.**	**Poll.**	**Trans.**	**Poll.**	**Trans.**	**Poll.**	**Trans.**	**Poll.**	No	**Poll.**	**Trans.**
Qualiagro	Ct	UnP	No	UnP	**Trans.**	UnP	No	UnP	No	UnP	No	UnP	No
	FYM	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
	BIOW	UnP	**Trans.**	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
	GWS	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
	MSW	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
Yvetot	RP1	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
	RP2	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
	PT2	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
	PT1	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
	PP	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
	GC	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
BioREco	OG_A	UnP	No	UnP	No	UnP	No	UnP	No	**Poll.**	No	UnP	No
	OG_GD	UnP	No	UnP	No	UnP	No	UnP	No	**Poll.**	**Trans.**	UnP	No
	LI_A	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
	LI_GD	UnP	No	UnP	No	UnP	No	UnP	No	**Poll.**	No	UnP	No
	CV_A	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
	CV-GD	UnP	No	UnP	No	UnP	No	UnP	No	**Poll.**	No	UnP	No
Thil	ST	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
	SMP	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
	MP	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
	RT	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
RENECOFOR	F08	UnP	No	**Poll.**	**Trans.**	**Poll.**	No	UnP	No	UnP	No	UnP	No
	F57	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
	F76	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No
	F63	UnP	No	UnP	No	UnP	No	UnP	No	UnP	No	**Poll.**	**Trans.**
Polluted site presenting a Transfer of metal		70.6%		68.8%		42.9%		50.0%		5.56%		29.4%	
Unpolluted site presenting a Transfer of metal		3.85%		7.41%		6.90%		0%		0%		0%	

Soil: a soil with total metal concentration above the threshold value is identified by "Poll."

Metal Transfer: a plot presenting a metal transfer (median of snails' metal concentration above the CIRef value) is identified by "Trans."

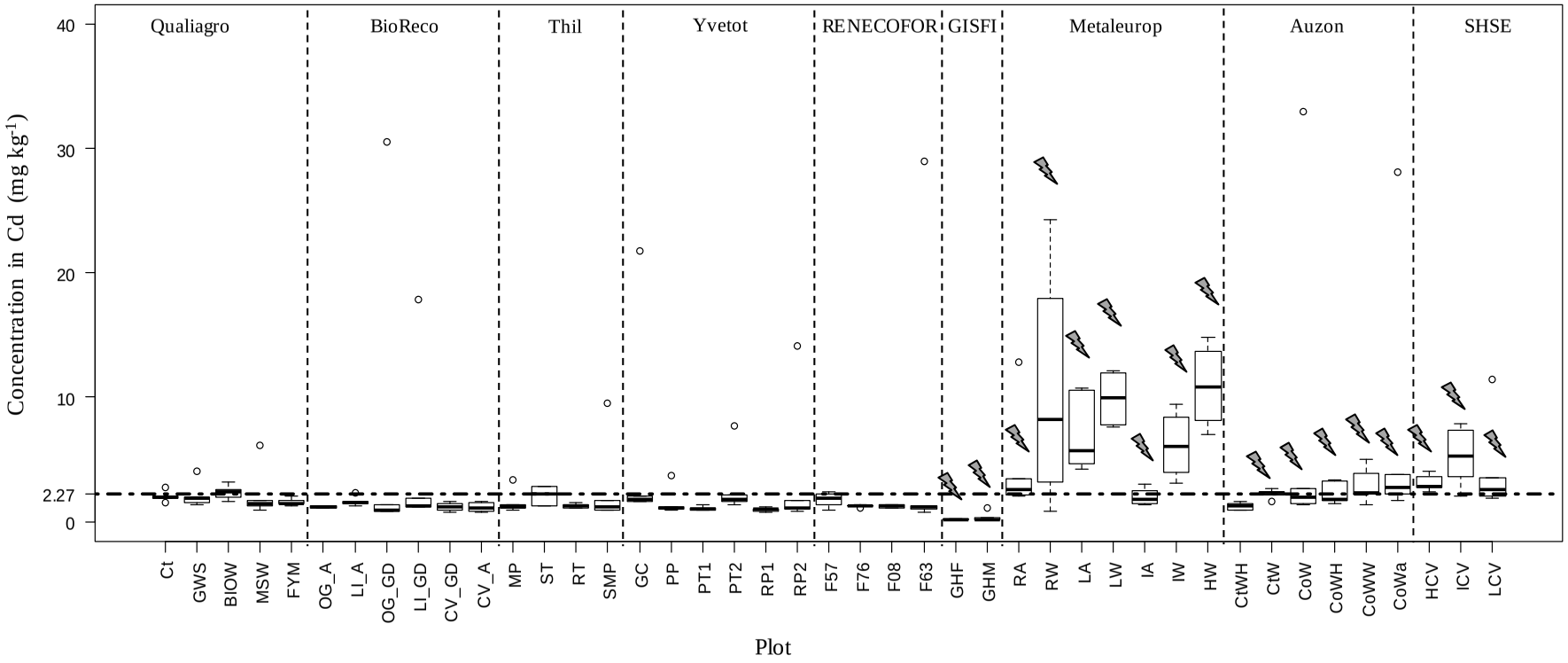

Figure 33: Median Cd concentration in snails after 28 days at the sites. The dot-dashed line represents the Cd CIRef. A lightning bolt identifies a Cd-polluted soil (i.e., containing more than 0.45 mg Cd kg^{-1}).

Table 10: Mono and multivariate regression formulas relating the accumulation of metals to total soil concentration and to total soil concentration coupled to soil characteristics.

Metal	Regression equation	*p-value*	R^2_{adj}
Cd	$\log(Cd_{sn}+1) = 0.355^{***} + 0.357 \log(Cd_{soil}+1)^{***}$	<0.001	0.43
	$\log(Cd_{sn}+1) = 0.629^{***} + 0.411 \log(Cd_{soil}+1)^{***} - 0.208 \log(C_{org}+1)^{**}$	<0.001	0.51
Pb	$\log(Pb_{sn}+1) = 0.378^{**} + 0.324 \log(Pb_{soil}+1)^{***}$	<0.001	0.37
	$\log(Pb_{sn}+1) = -0.033 + 0.353 \log(Pb_{soil}+1)^{***} + 0.095\ pH^{**} - 0.345 \log(C_{org}+1)^{*} + 22.4 \log(Fe_{ox}+1)^{**}$	<0.001	0.56
As	$\log(As_{sn}+1) = -0.122^{***} + 0.158 \log(As_{soil}+1)^{***}$	<0.001	0.67
	No significant improve with soil characteristics		
Cr	$\log(Cr_{sn}+1) = -0.021 + 0.145 \log(Cr_{soil}+1)^{**}$	0.001	0.20
	$\log(Cr_{sn}+1) = 0.553^{**} + 0.142 \log(Cr_{soil}+1)^{***} + 0.132 \log(C_{org}+1)^{*} - 0.411 \log(CEC+1)^{***} - 0.131 \log(sands+1)^{*}$	<0.001	0.41
Cu	$\log(Cu_{sn}+1) = 1.92^{***} + 0.085 \log(Cu_{soil}+1)^{*}$	0.027	0.09
	$\log(Cu_{sn}+1) = 1.79^{***} + 0.070 \log(Cu_{soil}+1)^{*} + 0.495 \log(clay+1)^{***} - 0.187 \log(Al_{ox}+1)^{*} - 0.350 \log(silts+1)^{***}$	<0.001	0.47
Zn	$\log(Zn_{sn}+1) = 2.79^{***} + 0.097 \log(Zn_{soil}+1)^{**}$	0.003	0.17
	$\log(Zn_{sn}+1) = 2.44^{***} + 0.149 \log(Zn_{soil}+1)^{***} - 0.151 \log(C_{org}+1)^{*} + 0.286 \log(Al_{ox}+1)^{**} + 0.172 \log(sands+1)^{***}$	<0.001	0.44

Table 11: Ranking of the sites by SET according to their risk of metal transfer. Values in bold correspond to AQ>1 and SET>0.

Site	Plots	QA_{Cd}	QA_{Pb}	QA_{As}	QA_{Cr}	QA_{Cu}	QA_{Zn}	SET_{plot}	SET_{site}
Metaleurop	HW	**4.804**	**8.666**	1.000	1.000	1.000	1.000	**11.470**	**4.261**
	IW	**2.658**	**2.693**	1.000	1.000	1.000	1.000	**3.350**	
	IA	1.000	**1.106**	1.000	1.000	1.000	1.000	**0.106**	
	LW	**4.381**	**3.768**	1.000	1.000	1.000	1.000	**6.149**	
	LA	**2.525**	**4.751**	1.000	1.000	1.000	**1.073**	**5.349**	
	RW	**3.632**	**1.068**	**1.202**	1.000	1.000	**1.108**	**3.009**	
	RA	**1.146**	1.000	**1.248**	1.000	1.000	1.000	**0.393**	
Auzon	CoWW	**1.005**	1.000	**6.433**	1.000	1.000	1.000	**5.438**	**3.206**
	CoW	1.000	1.000	**1.860**	1.000	1.000	1.000	**0.860**	
	CoWH	1.000	1.000	**4.332**	1.000	1.000	1.000	**3.332**	
	CoWa	**1.212**	**1.099**	**3.974**	1.000	1.000	1.000	**3.285**	
	CtW	**1.022**	1.000	**7.296**	1.000	1.000	1.000	**6.319**	
	CtWH	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
SHSE	HCV	**1.252**	**1.099**	1.000	**1.162**	1.000	1.000	**0.512**	**2.259**
	ICV	**2.305**	**3.258**	1.000	1.000	1.000	**1.129**	**3.692**	
	LCV	**1.128**	**2.027**	**1.844**	**1.291**	1.000	**1.283**	**2.574**	
RENECOFOR	F08	1.000	**3.915**	1.000	1.000	1.000	1.000	**2.915**	**0.732**
	F57	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
	F76	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
	F63	1.000	1.000	1.000	1.000	1.000	**1.011**	**0.011**	
Qualiagro	Ct	1.000	**1.052**	1.000	1.000	1.000	1.000	**0.052**	**0.018**
	FYM	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
	BIOW	**1.040**	1.000	1.000	1.000	1.000	1.000	**0.040**	
	GWS	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
	MSW	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
GISFI	GHF	1.000	1.000	1.000	1.000	1.000	1.000	0.000	**0.011**
	GHM	1.000	**1.021**	1.000	1.000	1.000	1.000	**0.021**	
BioREco	OG_A	1.000	1.000	1.000	1.000	1.000	1.000	0.000	**0.003**
	OG_GD	1.000	1.000	1.000	1.000	**1.017**	1.000	**0.017**	
	LI_A	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
	LI_GD	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
	CV_A	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
	CV-GD	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
Thil	ST	1.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000
	SMP	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
	MP	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
	RT	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
Yvetot	RP1	1.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000
	RP2	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
	PT2	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
	PT1	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
	PP	1.000	1.000	1.000	1.000	1.000	1.000	0.000	
	GC	1.000	1.000	1.000	1.000	1.000	1.000	0.000	

III.5. The SET approach for ranking sites and plots

The accumulation quotients (AQ) and the sums of excess of transfer (SET) in each plot and site are presented in Table 11. SET index identifies and quantifies metal transfer in each plot (SET_{plot}) at each site and allows plots and sites to be ranked according to their management priorities. The sites of Metaleurop, Auzon and SHSE exhibited the highest metal transfers (SET_{site}= 4.26, 3.21 and 2.26, respectively). The SET_{plot} index showed that the most polluted plots were not the ones that had the highest metal transfers (e.g., plots IA and CoW). This method also identifies which metals were accumulated by the snails. Indeed, at Metaleurop, both Cd and Pb were mainly transferred, whereas at Auzon, As was transferred the most. At the SHSE site, Cd and Pb and, to a lesser extent, As and Cr were accumulated by snails. The RENECOFOR site exhibited Pb transfer in plot F08. No metal transfer in the plots at the GISFI site was evidenced, even though its soils are polluted; only a slight Pb transfer occurred in the GHM plot. At the farm sites, little or no metal transfer was observed (SET_{site}= 0.018, 0.003, 0 and 0 for Qualiagro, BioREco, Yvetot and Thil, respectively), putting these sites at the bottom of the priority list for soil rehabilitation.

IV. Discussion

IV.1. Influence of soil properties on snails metal accumulation

The present study establishes for the first time the influence of total soil metal concentration and soil properties on snail accumulation of Cd, Pb, As, Cr, Cu and Zn *in situ*. Transfer to snails is mainly interpreted here using soil parameters as a global indicator of the quality of the environment. Even if the specific vegetation (itself influenced by soil parameters) may help to improve the relationship between soil and snail contaminations, this exposure source of snails was not considered here as the purpose was to set a simplified approach based on CIRef/SET calculation.

We found that total soil metal concentration accounts for more than 37% of the Cd, Pb and As accumulated by snails, but explains less than 20% of Cr, Cu and Zn accumulation. Except for As, the addition of soil parameters improves the explanation of metal accumulation by snails. This underlines the necessity to use bioindicators and to consider other parameters, such as speciation and soil characteristics, for accurate bioavailability assessment.

We also identified a number of polluted plots (n= 5 for Cd and Pb) in which no excessive metal transfer to snails was noted. This could partly be explained by the influence of soil properties on the bioavailability of metals (Pauget et al., 2011). Indeed, all these plots had high organic carbon contents, a parameter known to decrease metal mobility by specific association with soil constituents (Sterckeman et al., 2004) and metal bioavailability for soil organisms, such as springtail (Nursita et al., 2009) or earthworm (Saxe et al., 2001; Peijnenburg and Jager, 2003b). Thus, both total metal concentrations in soils and soil properties must be taken into account to assess metal transfer.

For Cr, we focused on soil properties to explain the lack of metal transfer observed in polluted plots. However, the CEC and sand content (properties identified by multivariate regression as decreasing Cr transfer) in soil where no transfer is observed are low and thus do not explain the lack of Cr transfer. Other parameters must be taken into account, such as metal partitioning and redox equilibria that modify the mobility of metal in the soil and then its bioavailability to organisms. Indeed, Cr is mainly present in soil in its trivalent form (Cr(III)), which has a lower mobility than Cr(VI) (Tian et al., 2010). It has also been shown (Sun et al., 2009) that the reduction of Cr(VI) (the most toxic form) to Cr (III) by organic acids in soil is extremely fast, decreasing its bioavailability. This might

explain why Cr is not greatly accumulated in the viscera, suggesting low bioavailability of Cr to snails; a similar result was found for *Eisenia andrei* by Janssen et al. (Janssen et al., 1997b).
The main parameter that influences As accumulation is its total concentration in the soil, as shown by the monovariate equation; no influence of the soil properties were identified. This may be because, as with Cr, As mobility is highly conditioned by its speciation. Indeed, in normal redox conditions, As is mainly present in the soil in its pentavalent form (Alloway, 1995), which is less mobile and thus less bioavailable than the trivalent form. Maximal As adsorption onto soil particles has been seen in a range of pH frequently observed in soils (between 3 and 10 for As(III) and between 3 and 7.5 for As(V)) (Raven et al., 1998); this explains the lack of influence of pH on As accumulation in snails exposed to the soils in our study, as our soil pH ranges from 3.76 to 8.70. In the same way, the lack of influence of Al oxide content on As accumulation in snails that we observed could be related to the fact that the As complexation by the aluminum oxides do not depend on the amount of aluminum oxide content (Goldberg et al., 2001).

For Cu and Zn, we found that accumulation was not related to total soil concentration and only moderately explained by soil properties. This is most likely because both Cu and Zn are essential metals and are thus regulated and detoxified well by snails when present in excess in the environment (Dallinger et al., 1993).

IV.2. Use of CIRef and implication in risk assessment

The CIRef we selected among those available were the most restrictive in order to identify maximum transfer. Therefore, the CIRef must be considered carefully when the internal metal concentrations in the snails are close to the CIRef value. Indeed, by using a penalizing value, we unexpectedly highlighted low metal transfer on unpolluted sites, such as in the BIOW plot at the Qualiagro site. However, we believe that missing a metal transfer (false negative) is more problematic than finding a metal transfer in an unpolluted site (false positive) in the framework of site management procedures.

In most of the polluted sites, defined according to the soil threshold values, the use of CIRef allows for the identification of excessive metal transfer to snails. More unexpectedly, the CIRef also revealed a transfer of As in two uncontaminated plots at the Metaleurop site and of Pb in plot F08. This highlights the utility of active biomonitoring and the suitability of CIRef not only to assess metal bioavailability to snails, but also to obtain data on the exposure of consumers of snails, including humans in some countries. CIRefs can also be useful in documenting the lack of information on current maximum levels of metals in foodstuff, as snails are still being assimilated to marine mollusks (The commission of the European communities, 2006). The Cd-CIRef is lower than the legal consumption values in foodstuffs in Europe (1 mg_{metal} kg^{-1} wet wt ≈ 5 mg_{metal} kg^{-1} DW), whereas the Pb-CIRef is higher (7.5 mg_{metal} kg^{-1} dry weight for Pb) (The commission of the European communities, 2006). This implies that even if snails are collected on unpolluted sites, their viscera can contain metal concentrations above the legal values of foodstuffs as measured for Pb in the control plot of the Qualiagro site.

IV.3. The sum of Excess of Transfer (SET) methodology

The SET methodology, based on the comparison between the CIRef and the metal concentrations in a ubiquitous snail species exposed on site (to soil, vegetation, in natural environmental conditions), allows the calculation of a specific index (i.e., SET_{plot} or SET_{site}) to rank the plots or sites according to their management priorities. This index quantifies the intensity of the excess of global

metal transfer calculated, in our study, on the basis of 3 metals, 1 metalloid and 2 essential metals. The application of this methodology to the 12 sites of the Bioindicator program ranks the sites principally according to their soil contamination. At the top of the priorities list, we listed the industrially impacted sites according to their SET_{site} for Cd, Pb and As at Metaleurop, As at Auzon and Cd, Pb, As, Cr and Zn at SHSE. SET identifies the sites that present the highest metal transfer to snails by quantifying the intensity of transfer and determines which metals are concerned. These data (QA_{metal} and SET_{plot}) can be useful for the rehabilitation of a site. Many studies have noted the importance of taking into account the characteristics of the site, the type of pollutants (Mulligan et al., 2001), and the soil type (Gusiatin and Klimiuk, 2012) for site remediation. The SET tool will be relevant to evaluate the efficiency of site remediation by comparing the rank before and after treatment and to compare the efficiency of different remediation methods.

The SET methodology identified one polluted site (GISFI) as exhibiting no metal transfer, and this underlines the relevance of the real measurement of bioavailability. Here, snails act as biointegrators, reflecting the influence of the soil parameters such as organic carbon content, but also metal speciation (especially for Cr and As) and biological processes such as metal regulation on metal bioavailability. This methodology provides specific indexes (SET_{plot} or SET_{site}) to assess environment quality and to raise the alarm concerning the zooavailability of contaminants. A future goal for CIRef and SET is to enlarge the list of metals and contaminants usable for this new approach of site investigation. In the framework of the Bioindicators program, comparing the rank of a site in terms of metal transfer to snails will be made with other organisms, such as plants. Moreover, other endpoints (e.g., species diversity and the abundance of microarthropods, nematodes or earthworms) will be investigated to allow for the determination of relevant batteries of bioindicators for accurate soil quality and risk assessment.

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Chapitre 3 : Assessing the *in situ* bioavailability of trace elements to snails using accumulation kinetics

Abstract
The bioavailability of trace elements in soils is conditioned by both physico-chemical and biological parameters. In this study, the accumulation kinetics of cadmium (Cd), lead (Pb), arsenic (As) and antimony (Sb) were determined for 3 industrially impacted sites to assess the bioavailability of these contaminants to snails. Mono and multivariate regressions allowed the identification of CEC, silts and organic carbon content as the soil parameters modulating the *in situ* bioavailability of Cd and Pb. For all elements, the total concentrations in the soils were not good predictor (not significant correlation) of the bioavailability to snails. The Cd, As and Sb assimilation fluxes were correlated with the $CaCl_2$ extract concentrations, but this correlation was not observed with Pb. The total soil concentration coupled with soil properties best explained the variation in Pb assimilation, whereas their influences on Cd bioavailability were lower, signifying that other parameters such as contamination sources may modulate Cd bioavailability. Here, the As and Sb *in situ* accumulation kinetics are described for the first time and highlighted a slight bioavailability to snails at the studied sites. The absence of a correlation between the As or Sb assimilation fluxes and total metals in the soil coupled with the absence of influence of soil properties on their bioavailability may result from the speciation of these metalloids, which are known to modulate their mobility in soils. This study highlights the need to consider both physico-chemical and biological aspects of metal and metalloid bioavailability to assess the risk of metal transfer from soil to organisms.

Résumé :
La biodisponibilité des métaux est conditionnée tant par des paramètres physico-chimiques que biologiques. Dans cette étude, les cinétiques d'accumulation de deux métaux (Cd et Pb) et de deux métalloïdes ont été modélisées pour estimer la biodisponibilité de ces éléments aux escargots sur trois sites impactés par des industries. Les équations mono- et multivariées ont permis de mettre en évidence l'influence de la CEC, des limons et du taux de carbone organique sur la biodisponibilité du Cd et du Pb, tandis que pour l'As et le Sb aucune influence des paramètres des sols n'a pu être mise en évidence. Pour les quatre éléments, les concentrations totales en métaux du sol ne sont des mesures pleinement satisfaisantes de leur biodisponibilité aux escargots bien que les concentrations en Cd, As et Sb extraite au $CaCl_2$ soient corrélées aux flux d'assimilation de ces trois éléments. Le couplage de la concentration totale et des paramètres des sols permet d'expliquer la majorité des variations des flux d'assimilation du Pb tandis que pour le Cd cette explication est plus faible. Ceci laisse supposer que d'autres paramètres entrent en jeu dans la modulation de la biodisponibilité du Cd comme les sources de contamination. Les cinétiques d'accumulation de l'As et du Sb sont décrites pour la première fois *in situ*. Une faible biodisponibilité de ces deux métalloïdes aux escargots a été mise en évidence sur les trois sites étudiés. L'absence d'influence des propriétés du sol sur la biodisponibilité de l'As et du Sb couplé à l'absence de corrélation entre leurs flux d'assimilation et la concentration totale du sol peut résulter de la spéciation de ces métalloïdes, la spéciation étant connue pour moduler la mobilité dans les sols. Cette étude met en évidence la nécessité de prendre en compte à la fois les aspects physico-chimiques et biologiques des la biodisponibilité des contaminants pour évaluer leur risque de transfert du sol aux organismes.

I. Introduction

In France, many sites present soil contaminated by metals and metalloids due to anthropogenic activities such as soil mining (Matera et al., 2003; Gis Sol, 2011). Currently, risk assessment procedures often ignore the soil characteristics, although these characteristics may influence both metal mobility in soil (Young et al., 2000) and the transfer of the contaminants to primary producers and primary consumers (Kabata-Pendias, 2004). This transfer corresponds to the flux of a pollutant from a compartment (biotic or abiotic) to organisms and is conditioned by trace element exposure and bioavailability. To analyze the risk of metal transfer, metal bioavailability must be estimated using biological measures (ISO 17402, 2008).

For this purpose, the measurement of metal accumulation kinetics in bioindicators that consider the dynamic processes of bioavailability is an efficient method (Gimbert et al., 2006; Van Straalen et al., 2008a; Pauget et al., 2011). Among the panel of bioindicators, the garden snail (*Cantareus aspersus)*, already used in active biomonitoring (Fritsch et al., 2011) and for metal bioavailability assessment (Coeurdassier et al., 2010), presents numerous advantages: it lives at the soil-plant-air interface and then integrates the different sources (soil, plant…) and routes (dermal and digestive) of contamination (Coeurdassier et al., 2002; de Vaufleury et al., 2006; Scheifler et al., 2006).

Currently, much work is being performed to find chemical extractants that allow the bioavailable pool of contaminants to be mimicked (ISO 17402, 2008; Denys et al., 2009a; Caboche et al., 2010). Assessing metal bioavailability using accumulation kinetics requires the lethal measurement of metal concentration in tissues. To eliminate the sacrifice of organisms and to determine the effectiveness of the mimetic chemical method of metal bioavailability, an evaluation of the ability of chemical methods to assess metal bioavailability to snails must be conducted. Calcium chloride ($CaCl_2$) extraction has been determined as usable for the assessment of the phytoavailability and bioavailability of metals to soil organisms (ISO 17402, 2008). However, the inability of $CaCl_2$ extract to assess the bioavailability of Cd and Pb to snails has been highlighted (Fritsch et al., 2011; Mourier et al., 2011; Pauget et al., 2011). The correlation between the metal extracted concentration and the *in situ* metal bioavailability to *C. aspersus* determined by active monitoring with consideration of the dynamic processes of bioavailability has never been studied. Accordingly, the objective of this study was first to evaluate the bioavailability of two metals (cadmium (Cd) and lead (Pb)) and, for the first time, two metalloids (arsenic (As) and antimony (Sb)) *in situ* at three polluted sites on the basis of their accumulation kinetics and the calculation of their assimilation flux (a). Another objective was to estimate the influence of *in situ* soil properties on metal bioavailability using multivariate equations. We also investigated whether the total soil concentration or $CaCl_2$ extract of metals could be used to assess the bioavailability of these 4 trace elements to snails.

II. Materials and method

II.1. Site characteristics

Three industrial sites from among those of the Bioindicator 2 program (Pérès et al., 2011) were studied (the soil characteristics of each plot are presented in Table 12):

The Metaleurop site (a contaminated site) is located in northern France (50°29’ N, 2°59’E) and extends around the former lead smelter of Noyelles–Godault, closed in 2003. Four areas were selected according to a metal pollution gradient in the vicinity (H, I and L for high, intermediate and low, respectively) and at 10 km (R for reference) from the former smelter, as well as two areas that

Table 12: Medians of the physico-chemical characteristics of the three studied sites of the Bioindicator program. ***Control plots are in italics.***

Site	Plot	pH_w	Silts ($g\ kg^{-1}$)	Sands ($g\ kg^{-1}$)	OM ($g\ kg^{-1}$)	Clay ($g\ kg^{-1}$)	CEC ($cmol\ kg^{-1}$)	C_{org} ($g\ kg^{-1}$)	$[Al]_{ox}$ ($cmol\ kg^{-1}$)	$[Fe]_{ox}$ ($cmol\ kg^{-1}$)	$[Cd]_{tot}$ ($mg\ kg^{-1}$)	$[Pb]_{tot}$ ($mg\ kg^{-1}$)	$[As]_{tot}$ ($mg\ kg^{-1}$)	$[Sb]_{tot}$ ($mg\ kg^{-1}$)	$[Cd]_{CaCl2}$ ($mg\ kg^{-1}$)	$[Pb]_{CaCl2}$ ($mg\ kg^{-1}$)
Metaleurop	HW	7.99	601	101	83.5	298	29.3	48.3	0.038	0.014	34.4	2485	39.3		0.415	0.465
	IW	8.19	609	96.0	46.0	294	24.3	26.6	0.040	0.020	13.3	731	30.9		0.079	0.039
	IA	8.21	474	211	45.1	318	29.3	26.1	0.054	0.014	8.54	482	18.1		0.066	0.031
	LW	6.45	283	517	55.0	203	17.2	31.8	0.059	0.015	5.44	319	9.42		0.578	0.142
	LA	7.90	366	452	29.3	181	13.2	16.9	0.078	0.020	3.12	143	8.67		0.047	0.009
	RW	*6.55*	*525*	*313*	*34.9*	*163*	*12.2*	*20.2*	*0.053*	*0.011*	*1.09*	*48.8*	*7.11*		*0.122*	*0.011*
	RA	*6.96*	*531*	*290*	*25.5*	*181*	*13.0*	*14.7*	*0.048*	*0.010*	*0.968*	*41.7*	*8.60*		*0.045*	*0.005*
Auzon	CoWW	5.41	427	346	103	238	19.1	59.6	0.425	0.015	9.97	4575	3285	3930	0.326	0.617
	CoW	5.73	279	574	70.6	148	13.0	40.8	0.176	0.016	0.722	104	339	37.8	0.054	0.019
	CoWH	5.23	167	757	76.5	89.5	11.0	44.2	0.410	0.017	1.32	282	661	176	0.120	0.194
	CoWa	5.81	167	713	60.3	98.5	9.46	34.9	0.111	0.009	6.74	1834	1087	2221	0.219	0.377
	CtW	*6.22*	*299*	*556*	*70.7*	*138*	*14.9*	*40.9*	*0.069*	*0.012*	*0.612*	*60.1*	*123*	*11.9*	*0.034*	*0.004*
	CtWH	*5.10*	*225*	*628*	*45.2*	*148*	*8.90*	*26.2*	*0.814*	*0.016*	*0.143*	*28.1*	*62.5*	*9.93*	*0.021*	*0.021*
SHSE	HCV	8.10	142	803	81.1	48.5	10.5	46.9	0.081	0.030	21.0	2525	73.3		0.018	0.027
	ICV	8.74	131	820	52.5	44.5	6.60	30.4	0.065	0.026	9.37	1616	54.8		0.004	0.012
	LCV	8.57	77	886	25.6	33.5	3.47	14.8	0.070	0.031	1.99	513	30.4		0.001	0.004

had been used (woodland W and arable A) were studied. Due to French law, any arable plot must be located at least 0.5 km from the smelter leading to 7 plots (i.e. HW, IW, IA, LW, LA, RW and RA).
The Auzon site (45°23'12N, 03°21'32 E) is an industrial waste site with trace element contamination, such as arsenic. Six plots (4 contaminated "Co", 2 controls "Ct") were selected according to a metal pollution gradient (from 62 to 3600 $mg.kg^{-1}$ As) and vegetation cover (woodland "W", woodland on hydromorphic soil "WW", woodland hedge "WH" and wasteland "Wa").
The SHSE site is a metallurgical landfill near Saint-Etienne (45°43'N, 4°39'E), with high metal contamination combined with herbicide contamination. Three plots were selected according to their level of plant cover, with a gradient from the "high level of cover of vegetation" plot (HCV) to the "low level of cover of vegetation" plot (LCV).

II.2. Animals

Juvenile brown garden snails (*Cantareus aspersus aspersus* Müller, 1774, syn. *Helix aspersa aspersa*) were reared as described in ISO (ISO 15952, 2006) under controlled conditions. The individuals used for the test were subadults (n = 726) that were reared for 7 to 9 weeks, weighing 4.9±0.7 g; the mean shell diameter was 25.7±1.1 mm (mean±SD). The viscera concentrations of the snails before exposure were 0.73±0.10 mg kg_{sn}^{-1} for Cd, 0.59±0.26 mg kg_{sn}^{-1} for Pb, 0.33±0.11 mg kg_{sn}^{-1} for As and 0.03±0.004 mg kg_{sn}^{-1} for Sb.

II.3. Exposure design

At each plot, three microcosms (25 x 25 cm stainless steel cylinders) containing 15 snails each were arranged in a 1-m diameter circle. The snails were exposed to the soil and vegetation under natural climatic conditions (Scheifler et al., 2003a). After 0, 2, 5, 7, 14, 21 and 28 days, 2 snails were randomly sampled from each of the 3 microcosms of each plot.

II.4. Analysis

II.4.a. Soil

Total soil metal concentrations and concentrations in the $CaCl_2$ extracts were measured using inductively coupled plasma atomic emission spectrophotometry (ICP-AES). The soil analyses were performed by the *Soil Analysis laboratory of Arras* (France), which benefits from the COFRAC (French accreditation committee) accreditation n°1-1380 (available at www.cofrac.fr) for its analytical quality of metal measurements in soils.
More details regarding sampling strategy and analysis are presented in Pérès et al. (Pérès et al., 2011).

II.4.b. Animals

Following sampling, the snails were placed in clean containers, fasted for 48 h (the feces were removed after 24 h) and weighed. They were sacrificed by freezing at -80°C. After thawing, the entire soft body was removed from the shell, and the foot and the viscera were separated (Gomot-de Vaufleury and Pihan, 2002). In this study, we focused on the viscera because they contain the hepatopancreas (digestive gland), which is the primary site of metal accumulation and storage in snails (Hopkin, 1989). The viscera were oven-dried at 60°C until they reached a constant weight, then digested in nitric acid (HNO_3 65%, Carlo-Erba analytical quality) and analyzed using graphite furnace atomic absorption spectrometry (GFAAS) for the snails from Metaleurop and using ICP-AES for the

snails from Auzon and SHSE. The validity of the analytical methods was confirmed using standard biological reference material (TORT-2, lobster hepatopancreas; National Research Council of Canada–Institute for National Measurement Standards, Ottawa, ON, Canada). The average deviations from the certified values were 1.21±0.74% and 8.79±10.8% and 13.8±2.53 for Cd, Pb and As, respectively. The detection limits for the Metaleurop, Auzon and SHSE sites were 0.014, 0.002 and 0.010 mg kg_{sn}^{-1} for Cd, t0.342, 0.004 and 0.032 mg kg_{sn}^{-1} for Pb and 0.212, 0.010 and 0.026 mg kg_{sn}^{-1} for As, respectively. For Sb, the detection limits were 0.004 mg kg_{sn}^{-1}.

II.5. Statistical analysis and modeling

II.5.a. Static accumulation

The metal accumulation in snails was measured using the medians of the internal metal concentration in the snail viscera after 28 days of exposure ($C_{sn}(28)$). The bioaccumulation factor after 28 days of exposure (BAF_{28}) is calculated using the equation (Eq. 1):

(Eq. 1) $$BAF_{28} = \frac{C_{sn}(28)}{C_{tot}}$$

where $C_{sn}(28)$ is the internal concentration of metal in the snail viscera after 28 days of exposure and (C_{tot}) is the total concentration of the metal in soil.

II.5.b. Accumulation kinetics

A one-compartment model was used to fit the accumulation kinetics data (Gimbert et al., 2006; Pauget et al., 2011). This model expresses the dynamic change of the metal concentration in the tissues over time, following the equation (Eq. 2):

(Eq. 2) $$C_{sn}(t) = C_{sn}(0) + \frac{a}{k_2}\,(1 - e^{-k_2 * t})$$

where $C_{sn}(t)$ is the metal concentration in the snail viscera (mg_{metal}. kg DW_{sn}^{-1}, Dry Weight) at the time t (days); $C_{sn}(0)$ is the metal concentration measured in six snails at the beginning of the experiment; a is the assimilation flux constant (*i.e.,* metal crossing biological membranes), which is representative of bioavailability (mg_{metal}.kg DW_{sn}^{-1}.d^{-1}); and k_2 is the excretion rate constant (d^{-1}).

The accumulation and elimination parameters were estimated by fitting the models with a non-linear mixed-effects procedure (nlme) that allows nested random effects. The within-group errors were allowed to be correlated and/or to have unequal variances. The nlme integrates the soil as a fixed factor and the microcosm as a random effect. Statistical differences in the parameter estimates among treatments were judged from the absence of overlap of their 95% confidence intervals (95% CI).

II.5.c. Determination of the influence of soil parameters on bioavailability.

To determine the influence of soil parameters on bioavailability, multivariate regressions of bioavailability (a) against total soil concentration and soil parameters were performed according to (Peijnenburg et al., 1999c), using the equation (Eq. 3):

(Eq. 3) $$Y = x * A + y * B +, \ldots, z$$

where Y = log(a+1) or log(k_1+1); x, y, … are coefficients and A, B, … represent the pH_{water} and the log(parameter+1) of C_x (the total concentration in the soil or $CaCl_2$ extract, the clay contents, organic

carbon concentration (C_{org}), the cationic exchange capacity (CEC) and aluminum (Al_{ox}) and iron (Fe_{ox}) oxides). The best model was chosen based on the AICc (Burnham and Anderson, 2004).

II.5.d. Determination of the ability of total soil concentration or $CaCl_2$-extracted metal concentration to assess bioavailability

The efficiency of an extractant to assess bioavailability was checked by deriving an uptake rate constant (k_1), allowing the influence of the soil parameters to be compared with both bioavailability (a) and environmental availability (C_x) (Eq. 4):

$$k_{1(x)} = \frac{a}{C_x} \quad \text{(Eq. 4)}$$

The uptake rate (k_1) is expressed in kg_{soil}.kg DW_{sn}^{-1}.d^{-1}. The assimilation flux (a, mg_{metal}.kg DW_{sn}^{-1}.d^{-1}) is constant over the duration of exposure. (C_x) is estimated according to (1) the total soil concentration (C_{tot} in mg.kg DW_{soil}^{-1}) and (2) the $CaCl_2$-extractable metal concentration (C_{CaCl2} in mg.kg DW_{soil}^{-1}).

To determine whether a chemical method is usable to assess bioavailability, the uptake rates (k_1) were fitted against soil characteristics using multivariate regressions. As shown in Pauget et al. (Pauget et al., 2011), a chemical method is potentially usable when there are no significant regressions. Indeed, the absence of a significant influence of soil characteristics on uptake variation suggests that the influence of soil physico-chemical properties on the extractable concentration and on bioavailability is nearly similar. This absence does not means that environmental availability and bioavailability are necessarily influenced by the same parameters, but it indicates that the tested extractant may be a potential indicator of bioavailability. Following this first screening, the correlation between bioavailability (a) and the chemical estimation of environmental availability (C_x) was calculated using a monovariate regression to confirm the ability of these two chemical methods (total soil concentration or $CaCl_2$-extracted metal concentration) to assess the bioavailability of the trace elements to snails.

All statistics were performed using the free statistical software package R, version 2.10.1 (R Development Core Team, 2011).

III. Results

III.1. Determination of metal accumulation by snails and the bioaccumulation factor (BAF)

After 28 days of exposure, only 6.5% snails have died showing the absence of letality of contaminant ingestion. During the 28 days of exposure, the snails accumulated the 4 studied elements in all of the plots of the studied sites except within the control plot CtWH at the Auzon site. In this plot, the internal concentrations of As in the snails after 28 days of exposure were similar to the internal concentrations in the snails at the beginning of the experiment (0.255 vs. 0.330 $mg_{As}.kg_{sn}^{-1}$, respectively). At the end of the exposure, the concentrations in the snails ranged from 1.30 to 10.9 $mg.kg_{sn}^{-1}$ for Cd, from 2.35 to 112 $mg.kg_{sn}^{-1}$ for Pb, from 0.255 to 1.98 $mg.kg_{sn}^{-1}$ for As and from 0.063 to 0.733 $mg.kg_{sn}^{-1}$ for Sb (Table 13).

For Cd, 9 BAF values were higher than 1, with values ranging from 1.29 to 9.09, and 7 were below 1, with values ranging from 0.14 to 0.56. For the 3 other metals, all of the BAFs were lower than 1, with

values ranging from 0.002 to 0.429 for Pb, from 0.001 to 0.018 for As and from 0.0001 to 0.015 for Sb (Table 13).

Table 13: Concentrations in snail viscera after 28 days of exposure ($C_{sn}(28)$) and the bioaccumulation factor (BAF) and the kinetic parameter estimates for the accumulation of Cd, Pb, As and Sb in *C. aspersus* snails exposed to the plots of the three industrial sites. a: assimilation flux; k_2: excretion rate; 95%CI: 95% confidence interval; NQ: not quantified. *Control plots are in italics.*

Metal	Site	Plot	Measured	Calculated	Modeled					
			$C_{sn}(28)$ $mg_{ETM}\ kg_{sn}^{-1}$	BAF_{28}	a $mg_{ETM}.kg_{sn}^{-1}.d^{-1}$	95% CI	*p-value*	k_2	95% CI d^{-1}	*p-value*
Cd	Metaleurop	HW	10.9	0.317	0.310	0.212/0.407	0.006	0.020	-0.021/0.060	0.654
		IW	6.03	0.453	0.228	0.135/0.321	0.030	0.038	-0.012/0.088	0.479
		IA	1.76	0.206	0.059	0.033/0.084	0.040	0.036	-0.017/0.090	0.530
		LW	9.93	1.83	0.200	0.158/0.243	<0.001	-	-0.055/0.000	0.359
		LA	5.73	1.84	0.272	0.221/0.324	<0.001	0.016	-0.005/0.036	0.485
		RW	*8.24*	*7.56*	*0.397*	*0.289/0.505*	*0.002*	*0.072*	*0.030/0.115*	*0.123*
		RA	*2.60*	*2.69*	*0.055*	*0.039/0.071*	*0.003*	*0.001*	*-0.027/0.028*	*0.984*
	Auzon	CoWW	2.27	0.228	0.242	0.177/0.308	0.002	0.116	0.058/0.174	0.070
		CoW	2.01	2.78	0.085	0.054/0.115	0.014	0.044	-0.002/0.090	0.318
		CoWH	1.83	1.39	0.059	0.053/0.068	<0.001	0.014	-0.003/0.030	0.444
		CoWa	2.75	0.408	0.133	0.092/0.174	0.005	0.054	0.015/0.094	0.207
		CtW	*2.32*	*3.79*	*0.079*	*0.071/0.088*	*<0.001*	*0.003*	*-0.011/0.016*	*0.850*
		CtWH	*1.30*	*9.09*	*0.052*	*0.038/0.065*	*0.001*	*0.085*	*0.045/0.126*	*0.059*
	SHSE	HCV	2.84	0.135	0.021	0.014/0.028	0.010	-	-0.120/-0.077	<0.001
		ICV	5.23	0.558	0.094	0.065/0.122	0.004	-	-0.063/-0.015	0.141
		LCV	2.56	1.29	0.093	0.077/0.108	<0.001	-	-0.053/-0.014	0.117
Pb	Metaleurop	HW	112	0.045	4.57	3.41/5.73	<0.001	0.013	-0.01/0.036	0.602
		IW	34.8	0.048	3.53	2.20/4.86	0.020	0.119	0.046/0.192	0.138
		IA	14.3	0.030	3.92	2.48/5.37	0.018	0.183	0.091/0.275	0.075
		LW	48.7	0.153	4.49	3.14/5.84	0.004	0.074	0.029/0.119	0.132
		LA	61.4	0.429	4.94	4.08/5.79	<0.001	0.066	0.043/0.089	0.012
		RW	*13.8*	*0.283*	*NQ*	*NQ*	*NQ*	*NQ*	*NQ*	*NQ*
		RA	*9.95*	*0.239*	*NQ*	*NQ*	*NQ*	*NQ*	*NQ*	*NQ*
	Auzon	CoWW	7.48	0.002	0.840	0.737/0.943	<0.001	0.131	0.088/0.175	0.008
		CoW	2.85	0.027	0.109	0.084/0.133	<0.001	0.022	-0.008/0.052	0.500
		CoWH	4.01	0.014	0.315	0.242/0.389	<0.001	0.047	0.002/0.093	0.339
		CoWa	14.2	0.008	0.419	0.330/0.508	<0.001	-	-0.035/0.002	0.404
		CtW	*4.30*	*0.072*	*0.402*	*0.329/0.475*	*<0.001*	*0.097*	*0.069/0.126*	*0.003*
		CtWH	*2.35*	*0.084*	*0.537*	*0.362/0.712*	*0.007*	*0.360*	*0.160/0.560*	*0.102*
	SHSE	HCV	14.2	0.006	0.242	0.194/0.290	<0.001	-	-0.059/-0.016	0.109
		ICV	42.1	0.026	1.91	1.55/2.28	<0.001	-	-0.066/0.001	0.367
		LCV	26.2	0.051	2.82	2.26/3.39	<0.001	0.067	0.032/0.102	0.082
As	Auzon	CoWW	1.98	0.001	0.744	0.628/0.860	<0.001	0.297	0.200/0.396	0.009
		CoW	0.571	0.002	0.022	0.012/0.032	0.041	0.058	-0.011/0.122	0.435
		CoWH	1.33	0.002	0.190	0.151/0.230	<0.001	0.026	-0.013/0.066	0.532
		CoWa	1.22	0.001	0.042	0.030/0.055	0.003	-	-0.051/0.007	0.489
		CtW	*2.24*	*0.018*	*0.378*	*0.330/0.426*	*<0.001*	*0.118*	*0.086/0.151*	*0.002*
		CtWH	*0.255*	*0.004*	*0.020*	*-0.004/0.045*	*0.449*	*0.270*	*-0.298/0.839*	*0.659*
Sb	Auzon	CoWW	0.495	0.00013	0.066	0.053/0.079	<0.001	0.131	0.056/0.206	0.117
		CoW	0.182	0.00481	0.011	0.009/0.013	<0.001	0.109	0.053/0.164	0.076
		CoWH	0.167	0.00095	0.021	0.018/0.024	<0.001	0.017	-0.022/0.055	0.690
		CoWa	0.733	0.00033	0.044	0.030/0.058	0.007	0.012	-0.027/0.051	0.779
		CtW	*0.183*	*0.01538*	*0.027*	*0.024/0.030*	*<0.001*	*0.237*	*0.189/0.285*	*<0.001*
		CtWH	*0.063*	*0.00634*	*0.028*	*0.015/0.041*	*0.057*	*0.903*	*0.421/1.39*	*0.090*

III.2. Determination of metal bioavailability to snails and excretion

Accumulation kinetics enabled the modeling of both the bioavailability (*a*: assimilation flux) and the excretion rate (k_2), which are two key parameters of transfer. For all of the elements, an increase in

the metal concentration in the snail viscera was observed during the exposure time (Figure 34) as shown by the significant assimilation fluxes, ranging from 0.021 to 0.397 mg.kg$_{sn}^{-1}$.d^{-1} for Cd, from 0.109 to 4.93 mg.kg$_{sn}^{-1}$.d^{-1} for Pb, from 0.022 to 0.744 mg.kg$_{sn}^{-1}$.d^{-1} for As and from 0.011 to 0.066 mg.kg$_{sn}^{-1}$.d^{-1} for Sb (Table 13). However, the Pb assimilation fluxes cannot be determined for the control plots RA and RW at the Metaleurop site, and the As assimilation flux was not significant (p-value=0.449) for the control plot CtWH of the Auzon site (Table 13). For Sb, the assimilation flux modeled for the control plot CtWH was the only one that was not significant (p-value=0.057). Two different accumulation patterns can be distinguished for the studied elements: a linear pattern when the metal was not excreted and a non-linear pattern leading to the stabilization of the internal concentration and the appearance of equilibrium when the metal was excreted by the snails (Figure 34). For Cd, no significant excretion was observed during the exposure time (Table 13). For Pb, only three significant excretion rates were determined for the plots LA (Metaleurop), CoWW and CtW (Auzon) (p-value: 0.012, 0.008 and 0.003, respectively). For As, two significant excretions rates were identified for the plots CoWW and CtW (p-value: 0.009 and 0.002, respectively), corresponding to the two higher As assimilation fluxes. For the plot CtW, a significant excretion of Sb was also identified (p-value < 0.001).

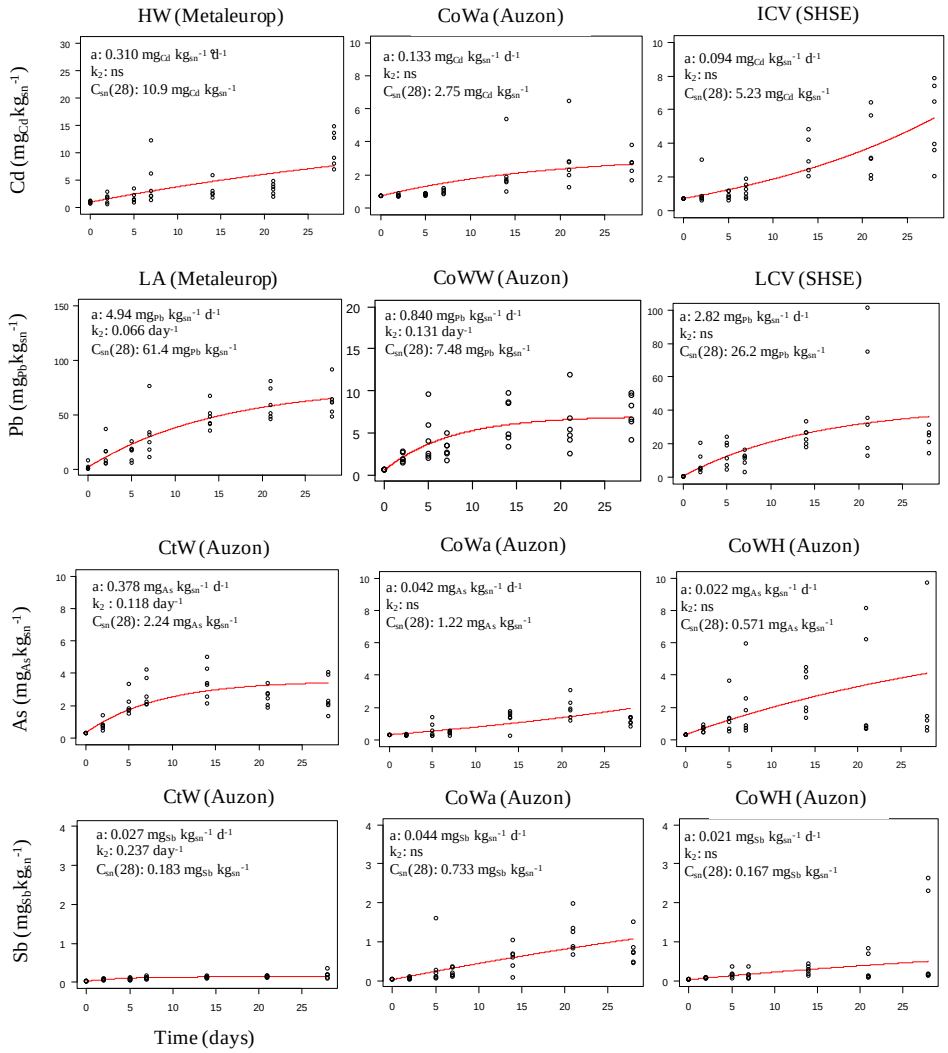

Figure 34: Accumulation kinetics of Cd, Pb, As and Sb in snails exposed to plots at the Metaleurop, Auzon and SHSE sites. Each data point represents an individual snail. a: assimilation flux; k_2: excretion rate; $C_{sn}(28)$: metal concentration in snail viscera after 28 days of exposure; ns: not significant.

III.3. Multivariate analysis correlating uptake rate and bioavailability to soil parameters

III.3.a. Influence of soil properties on metal bioavailability.

By correlating the assimilation fluxes to the total soil concentrations and soil parameters (Table 14), we identified the total concentrations, CEC (for Pb and Cd), silts (for Cd) and C_{org} content (for Pb) as parameters that modulate the bioavailability of Cd and Pb to snails. Indeed, the determination coefficients of the regressions (0.36 and 0.71 for Cd and Pb, respectively) coupled with the significant descriptors attest that the soil parameters influence the metal bioavailability to snails. For As and Sb, the soil properties do not influence their assimilation flux.

Table 14: Influence of the total soil concentration and soil parameters on the bioavailability of Cd, Pb, As and Sb (a, $mg_{metal}.kg_{sn}^{-1}.d^{-1}$) using multivariate regressions. Statistical significance: °: p-value<0.1; *: p-value<0.05; **: p-value<0.01; ***: p-value<0.001; ns: no significant regression.

Metal	Equation	*p-value*	r^2_{adj}
Cd	$\log(a_{Cd}+1) = -0.245^* + 0.042 \log(Cd_{soil}+1)^\circ - 0.172 \log(CEC+1)^\circ + 0.192 \log(silts+1)^*$	0.04	0.36
Pb	$\log(a_{Pb}+1) = 1.46^{**} + 0.176 \log(Pb_{soil}+1)^* + 0.814 \log(CEC+1)^{**} - 1.62 \log(C_{org}+1)^{***}$	0.001	0.71
As	$\log(a_{As}+1) = ns$		
Sb	$\log(a_{Sb}+1) = ns$		

III.3.b. Determination of the ability of the total metal soil concentration and the $CaCl_2$-extracted metal concentration to assess metal bioavailability to snails

First, we determined whether the soil parameters explained the variations in the uptake rates (k_1, Table 15). When using total soil concentration as an estimator of the environmental availability of Cd, a significant influence of silts, CEC and Al_{ox} was identified (p-value: 0.015, Table 15), suggesting that the total metal concentration was not usable to assess Cd bioavailability. For Pb, an influence of silts, sands and C_{org} content and sands was identified as modulating the uptake rate. As for Cd, this finding suggests that the total metal concentration in the soil seems to not be a usable method to assess Pb bioavailability to snails. For these two metals (Cd and Pb), the absence of correlations between the assimilation fluxes and the total soil concentrations (Table 15 and Figure 35) confirms that Cd and Pb bioavailability cannot be assessed by total metal concentration. For As and Sb, the variations in the uptake rates were not explained by measured soil parameters (*p-value* > 0.05), suggesting that the bioavailability of As and Sb may be assessed using total soil concentration (Table 15). However, the absence of correlations between the As and Sb assimilation fluxes and the total As and Sb concentrations in the soil refute this hypothesis (Table 15, Figure 35).

When the uptake rates were derived using the $CaCl_2$-extracted metal concentrations, the multivariate regressions highlighted an influence of pH (for Cd and Pb), CEC (for Cd) and C_{org} (for Pb) on the

variation in the uptake rates (k_1). The significance of these regressions leads to the supposition of the inefficiency of the 0.01M $CaCl_2$ extract to assess metal bioavailability. This hypothesis is confirmed for Pb with the absence of a correlation between the Pb-assimilation flux and the Pb-$CaCl_2$ extracted concentration, whereas for Cd, As and Sb, significant correlations were found (*p-value* = 0.042, 0.040 and 0.015, respectively), although a low determination coefficient was determined for Cd (r^2_{adj} = 0.21) (Table 15, Figure 35).

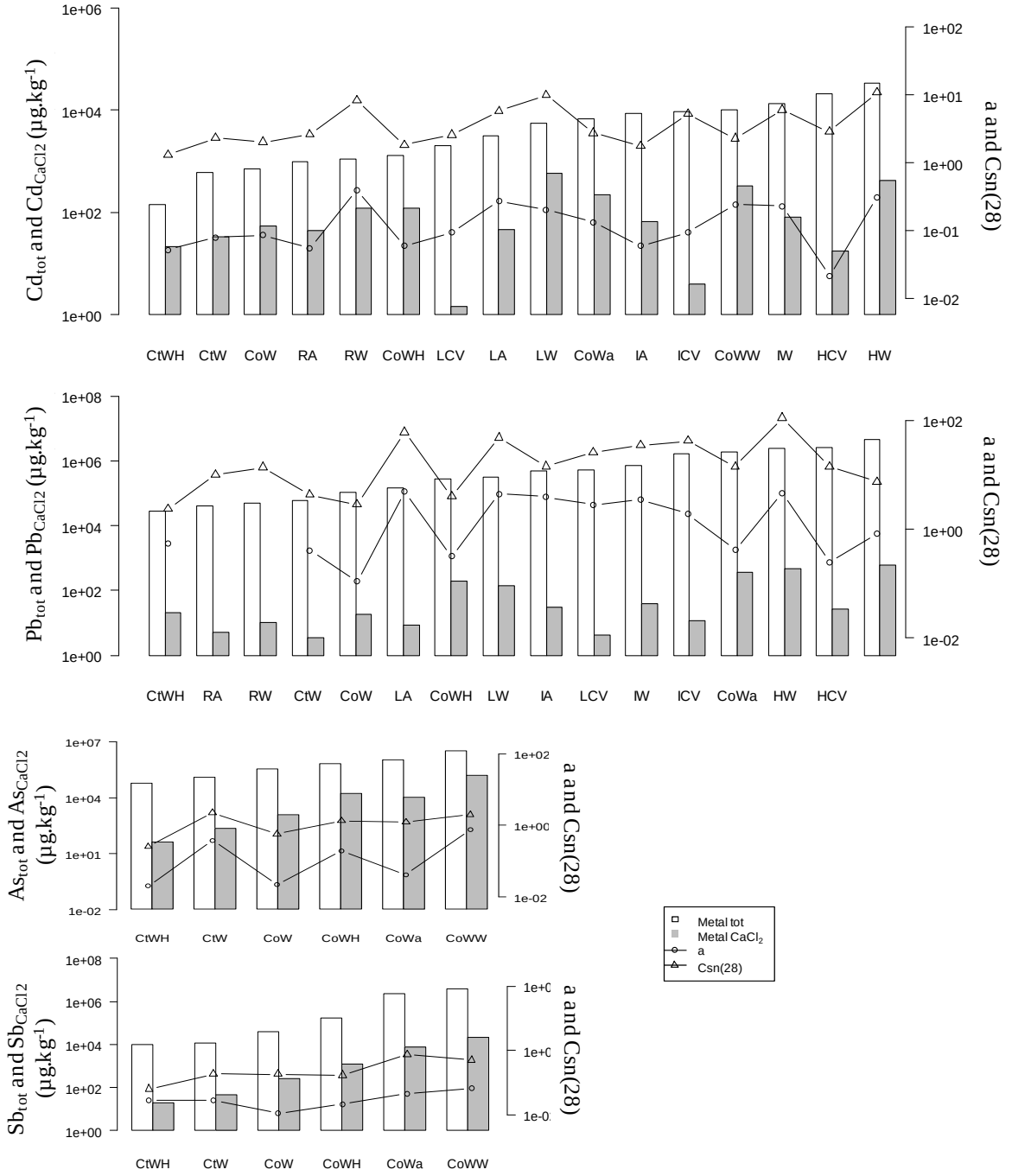

Figure 35: Comparison of accumulation in snail viscera after 28 days of exposure ($C_{sn}(28)$) and assimilation flux (a) with total soil metal concentration and $CaCl_2$-extracted metal concentration of Cd and Pb and of accumulation in snail viscera after 28 days of exposure ($C_{sn}(28)$) and assimilation flux (a) to total soil metal concentrations of As and Sb.

Table 15: Influence of (i) soil parameters on the uptake rate (k_1, $kg_{soil}.kg_{sn}^{-1}.d^{-1}$) estimated from the total metal concentration and the $CaCl_2$-extracted metal concentration and (ii) C_X on bioavailability using single relationship (monovariate regressions) for Cd, Pb, As and Sb. Statistical significance: °: p-value<0.1; *: p-value<0.05; **: p-value<0.01; ***: p-value<0.001; ns: no significant regression.

metal	item	method	equation	p-value	r^2adj	n
Cd	k_1~param	total	$\log(k_{1Cd}+1) = -0.180° + 0.183 \log(Silts+1)^{**} - 0.220 \log(CEC+1)^{*} + 0.285 \log(Al_{ox}+1)^{*}$	0.015	0.46	16
		$CaCl_2$	$\log(k_1+1) = -0.912° + 0.193\ pH^{**} + 1.49 \log(CEC+1)^{***}$	<0.001	0.74	
	a~C_x	total	$\log(a_{Cd}+1) = ns$			
		$CaCl_2$	$\log(a_{Cd}+1) = 0.040^{**} + 0.357 \log(Cd_{CaCl2}+1)^{*}$	0.042	0.21	
Pb	k_1~param	total	$\log(k_{1Pb}+1) = -0.056^{*} + 0.022 \log(Silts+1)^{**} + 0.015 \log(sands+1)^{**} - 0.021 \log(C_{org}+1)^{***}$	<0.001	0.74	14
		$CaCl_2$	$\log(k_{1Pb}+1) = 4.83^{*} + 0.239\ pH^{*} - 3.28 \log(C_{org}+1)^{**}$	<0.001	0.75	
	a~ C_x	total	$\log(a_{Pb}+1) = ns$			
		$CaCl_2$	$\log(a_{Pb}+1) = ns$			
As	k_1~param	total	$\log(k_{1As}+1) = ns$			5
		$CaCl_2$	$\log(k_{1As}+1) = ns$			
	a~ C_x	total	$\log(a_{As}+1) = ns$			
		$CaCl_2$	$\log(a_{As}+1) = -0.032 + 0.105 \log(As_{CaCl2}+1)^{*}$	0.04	0.74	
Sb	k_1~param	total	$\log(k_{1Sb}+1) = ns$			6
		$CaCl_2$	$\log(k_{1Sb}+1) = ns$			
	a~ Cx	total	$\log(a_{Sb}+1) = ns$			
		$CaCl_2$	$\log(a_{Sb}+1) = 0.008^{*} + 0.013 \log(Sb_{CaCl2}+1)^{*}$	0.015	0.76	

IV. Discussion

IV.1. Accumulation of Cd, Pb, As and Sb by snails

The metal concentrations in the snail viscera after 28 days of *in situ* exposure demonstrated that the snails accumulated the four studied elements. Although analyses have been conducted in previous studies under other experimental conditions for Cd, Pb and As (Gimbert et al., 2008a; Coeurdassier et al., 2010), the accumulation of Sb in snails is an novel finding. Here, for the first time, the measurement of the As and Sb accumulation kinetics in snails and the influence of soil characteristics on bioavailability were investigated *in situ*. When comparing the accumulated metal concentration in the snail viscera after 28 days of exposure with total soil contamination, we observed that the highest metal accumulations were not necessarily identified in the most contaminated plots (as observed for example in the plot LW for Cd).

The use of the metal bioaccumulation factor (BAF) permits the classification of snails as deconcentrators for Pb, As and Sb (BAF<1) according to the classification of Dallinger (Dallinger, 1993). For Cd, this classification remains unclear because of the great variability in the BAF values (6 less than 1, 4 between 1 and 2 and 5 greater than 2). This variability is surprising because snails have been classified as macroconcentrators for Cd in numerous studies (Dallinger, 1993; Scheifler et al., 2002). However, Gimbert et al. (Gimbert et al., 2008a) calculated kinetic Cd-BAF for snails less than 1

during a 56-day *in situ* experiment. The use of internal concentrations of metals, which may not have reached a steady state, to determine "static" BAF values for risk assessment purposes could lead to an underestimation of the metal accumulation. Indeed, in our study, only two (for Pb) and one (for Sb) BAF value were calculated on the basis of the steady-state internal metal concentrations; those were in plot CoWW for Pb ($C_{sn}(ss) = a/k_2 = 7.00\ mg_{Pb}\ kg_{sn}^{-1}$) and plot CtW for Pb and Sb ($C_{sn}(ss) = 4.73\ mg.kg_{sn}^{-1}$ and $0.141\ mg.kg_{sn}^{-1}$, respectively). For the other plots, where no excretion was highlighted by accumulation kinetics, the BAF could be greatly underestimated because of the absence of the achievement of steady-state internal concentration of metals in the snail viscera.

The kinetic studies that we performed allowed the consideration of the dynamic processes of accumulation and the determination of the two key parameters of bioaccumulation to be considered: the excretion rate (k_2) and the assimilation flux (a). The two different accumulation patterns (linear or non-linear tending to equilibrium) for the four elements observed were related to the excretion during the exposure time. The calculation of the excretion rates indicates that even if all of the studied metals were bioavailable to the snails, the specific physiological strategies for the different metals studied conditioned their subsequent fate in the tissues. Indeed, for Cd and Sb, the linear accumulation patterns lead to model no significant excretion (except in plot CtW). The non-linear accumulation patterns of Pb and As suggest that the excretion of these elements was observed in the plots that presented the highest bioavailability. Pb was significantly excreted in plots CoWW and CtW (Auzon), although lower Pb assimilation fluxes than those in Metaleurop were modeled. However, in these plots, we observed the highest assimilation fluxes of As coupled to significant As excretion. We can hypothesize that Pb excretion was induced by the As excretion. Indeed, these two metals have been determined as being part of the intermediate class by Nieboer and Richardson (Nieboer and Richardson, 1980), and this determination leads to the supposition these trace elements are subject to sequestration by the B-type granules (Hopkin, 1989), which can be excreted by snails. However, this sequestration of As by the granules may appear following the overload of the metallothioneins binding sites due to high As assimilation by snails, with As being mainly bound by the metallothioneins (Casado-Martinez et al., 2012).

BAF is a simple parameter that gives partial information; it can be useful for a first screening to determinate accumulation even if it does not provide information regarding the equilibrium of the internal concentration and the influence of contamination sources on trace element accumulation. Indeed, BAF_{28} is only based on soil concentration even if trace element accumulation may mainly due to plant ingestion (Scheifler et al., 2006). Thus, the dynamic processes of bioavailability should be considered during risk assessment procedures through the modeling of the excretion rate, which allows the steady-state concentration in snails to be determined, and the assimilation flux (dynamic processes that control the assimilation of the pollutant by the organism), which integrates the modulating parameters of bioavailability such as the influence of soil properties or contamination sources. The determination of the excretion rate allows the calculation of kinetic BAF at steady state concentration.

IV.2. Assessment of the assimilation flux of Cd, Pb, As and Sb and the influence of soil characteristics

In this study, we demonstrated that, even in the case of low metal concentrations in soil, snails are able to assimilate metals and allow the characterization of the metal bioavailability by the modeling of assimilation flux (a). We observed that the Sb bioavailability was very low compared with the

bioavailability of As and Pb. Indeed, even in plot CoWW, with high Sb contamination, the Sb assimilation flux was 10 times lower than those of Pb and As, which presented close concentrations in the soils. This result can be explained by the low mobility of Sb in the soil due to its adsorption on Al and Fe oxides (ATSDR, 1992; Murciego et al., 2007) leading to a reduced bioavailability of Sb (Denys et al., 2009a). For As, the assimilation flux values were in the same range as those of Pb with quite similar total soil concentrations, and the higher bioavailability of As compared with Sb may be attributed to its higher mobility in soil (Ettler et al., 2010; Ettler et al., 2012). The absence of an influence of soil characteristics on the bioavailability of As and Sb could be because their mobility is highly conditioned by their speciation. Indeed, in normal redox conditions, As and Sb are primarily present in the soil in pentavalent form (Alloway, 1995; Edwards et al., 1995), which is less mobile and thus less bioavailable than the trivalent form. The maximal adsorption of As on soil particles occurs within a range of pH frequently observed in soils (between 3 and 10 for As(III) and between 3 and 7.5 for As(V)) (Raven et al., 1998). Moreover, As adsorption onto aluminum oxides at all aluminum oxide concentrations (Goldberg et al., 2001) is a likely explanation for the absence of an influence of soil properties on As bioavailability. Similar retention mechanisms of Sb in the soil can also likely explain the absence of an influence of soil characteristics on Sb bioavailability (Wilson et al., 2010).

For Cd, the multivariate regression highlighted a slight influence of soil properties. The total Cd concentration in soil, the CEC and the silt content explained 36% of the variation in Cd assimilation flux, suggesting that parameters other than the tested ones (e.g., the ingestion of contaminated vegetation or humus) may influence the assimilation of Cd. Although CEC have been previously identified as modulating Cd bioavailability in a laboratory study (Pauget et al., *in review*), the presence of other contamination sources during the *in situ* experiment could greatly modify the importance of soil in the assimilation of Cd by snails; the primary source of Cd accumulation by snails has been identified as vegetation (Scheifler et al., 2006).

For Pb, a satisfactory assessment of its bioavailability to snails was obtained when using the total Pb concentration in the soil coupled with CEC and C_{org} content. Compared with Cd, this result suggests that the main contamination source of Pb is the soil, which is in accordance with Scheifler et al. (Scheifler et al., 2006), who found in a laboratory experiment that soil accounted for approximately 80% of the Pb accumulation in snails.

IV.3. Assessing bioavailability using chemical methods

For the 4 trace elements, the total soil concentration does not allow the assessment of the bioavailability to snails whereas it has been previously shown Cd and Pb strongly bound to soil particles may be assimilated by snails (Pauget et al., 2012). This underline the difficulty to extrapolate data obtained in laboratory studies to understand *in situ* bioavailability variation of trace metal. Indeed, the influence of contamination sources, climatic conditions may affect the trace element assimilation by snails during the exposure time. The correlation between the Cd, As and Sb bioavailability and the $CaCl_2$ extracted concentration's of these 3 elements s and the fact that 0.01M $CaCl_2$ extract being an good predictor of metal phytoavailability (Menzies et al., 2007) suggest that the accumulation of Cd, As and Sb is mainly due to the presence of contaminated vegetation as shown for Cd in the Part 3 Chapter 1. The importance of contamination sources have been already showed (Scheifler et al., 2006) on Cd accumulation by snails underlining the importance of considering all contamination sources during bioavailability assessment. It could also be explained by a close transfer of these 3 elements from soil to plants and from soil to snails (assimilated trace

elements coming mainly from the soil solution). The absence of correlation between Pb bioavailability and $CaCl_2$ extracted concentration may be due to the location of Pb on cell walls (Qureshi et al., 1986) which is slight bioavailable and to the fact that Pb is less mobile in soil than the other elements. With this consideration, it would be interesting to examine whether including the Cd and Pb concentration in the surrounding vegetation could improve the modeling of Cd uptake by snails.

Our methodology, based on the comparison of the influence of soil properties on both bioavailability and environmental availability via the variation in the uptake rates, concluded that the total metal concentration in soil could be a good predictor of As and Sb assimilation flux. However, the total soil concentration failed to assess bioavailability of theses 2 elements, as demonstrated by the absence of correlation. The As and Sb bioavailability were neither influenced by soil properties nor estimated by total metal concentration in the soil; thus, other parameters should modulate their assimilation, such as their speciation or the contamination sources. Studying the relationships among the bioavailability of As and Sb, their speciation and also the contamination of the surrounding vegetation may allow a better explanation of their assimilation.

The use of uptake rate is relevant to the identification of chemical methods with which to assess the bioavailability of metals to snails under controlled conditions, notably using soil as the only contamination source (Pauget et al., 2011), and when the speciation of the studied element is known. Here, we demonstrated that the use of uptake rates variation during an *in situ* experiment presenting multiple contamination sources seems to be less effective. Indeed, to calculate the uptake rate, the determination of a chemical method (C_x) that is representative of the real available pool, including all contamination sources, the element speciation, the influence of the soil characteristics on the mobility of the elements and the organism physiology, should be performed.

V. Conclusion

The *in situ* accumulation kinetics of As and Sb for snails have been described for the first time, showing a slight bioavailability of these metalloids to snails at the 3 studied sites. The importance of considering the influence of soil parameters and metal speciation during the assessment of bioavailability has been highlighted. The variability of soils in terms of ageing or metal mixture, environmental conditions and the different sources of exposure (e.g., vegetation, soil, or humus) causes the modeling of the influence of soil properties on metal bioavailability to be challenging. To clearly assess the metal bioavailability *in situ,* the sources of each assimilated metal must be better known. Moreover, a larger panel of chemical methods should be studied to determine a suitable estimator of the *in situ* bioavailability of trace elements to snails.

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Partie C : Expérimentation en laboratoire

Chapitre 1 : How contamination sources and soil properties can modify the metal bioavailability to snails.

Abstract:
To better understand the processes of metal bioavailability, numerous parameters have to be studied. Indeed, the different contamination sources for an organism and the soil properties influencing metal mobility in soil are important to consider because they modulate the metal bioavailability. To integrate all these influences, active biomonitoring is a relevant solution. Among the bioindicator of soil quality, the garden snail (Cantareus aspersus) integrates multiple sources (soil, plant…) and routes (digestive, dermal…) of contamination. However, the contribution of each source on metal bioavailability and the influence of the soil parameter on this contribution have never been studied with taking into account the dynamic process of bioavailability. The use of accumulation kinetics, allows the determination of assimilation flux of snails exposed to contaminated soil and/or contamination lettuce. This study showed that the main assimilation source of Cd was the lettuce (68%) whereas the one of Pb was the soil (90%). An increase of the plant contribution has been evidenced with a two-units soil pH decrease in relation with the increase of lettuce metal concentration. An increase of the soil contribution to metal assimilation has been highlighted with an increase of organic matter (OM) content in the soil probably in relation with an increase of soil consumption by snails. No clear excretion and influence of source on excretion has been modeled on the exposure and depuration duration for both metal. This study with the quantification of the contribution of sources on metal bioavailability may allow a better explanation of the processes which modulate the metal bioavailability.

Résumé:
Afin de mieux comprendre les mécanismes de la biodisponibilité des métaux, de nombreux paramètres doivent être étudiés. En effet, les différentes sources de contamination pour un organisme et les propriétés du sol qui influent sur la mobilité des métaux dans le sol sont importantes à prendre en compte lors d'évaluations de la biodisponibilité. Pour intégrer toutes ces influences, la biosurveillance active apparait comme pertinente. Parmi les bioindicateurs de la qualité des sols, l'escargot (*Cantareus aspersus*) intègre de multiples sources (sol, plantes ...) et voies (digestive, cutanée ...) de contamination. Cependant, la contribution de chaque source sur la biodisponibilité des métaux et l'influence des paramètres du sol sur cette contribution n'ont jamais été étudiés en tenant compte des processus dynamiques de la biodisponibilité. L'utilisation de cinétiques d'accumulation, a permis la détermination des flux d'assimilation des escargots exposés aux sols et/ou à de la laitue contaminés. Cette étude a montré que la source principale d'assimilation du Cd était la laitue (68%), tandis que celle de Pb était le sol (90%). Une augmentation de la contribution de la laitue a été mise en évidence avec une diminution de deux unités de pH du sol, en relation avec l'augmentation de la concentration en métal dans la laitue. Une augmentation de la contribution du sol à l'assimilation de métal a été mise en évidence due à une augmentation du taux de matière organique (MO) du sol, probablement en relation avec une augmentation de la consommation du sol par les escargots. Aucune excrétion nette ainsi que d'influence de la source de contamination sur l'excrétion n'a été modélisée sur la durée d'exposition pour les deux métaux. Cette étude, avec la quantification de la contribution des sources sur l'assimilation des métaux permettra une meilleure compréhension des processus qui modulent la biodisponibilité des métaux.

I. Introduction

Risk assessment methodology has been recognized as a powerful tool for the decision-making process in contaminated sites management, especially when contamination of soil or water is evidenced (Moreno-Jiménez et al., 2011). Due to the increase of contamination of the environment with metals since the onset of the industrial revolution, soil monitoring with bioindicators have been developed (Pérès et al., 2011). Usually, during ecological risk assessment (ERA), single-species tests which cover single and identified exposure source (*e.g.* soil, food…) or different routes (*e.g.* digestive, dermal…) associated with a single compartment (*e.g.* sediment, water….) are performed (Tarazona and Vega, 2002). However, during passive, *i.e.* naturally exposed sentinels species, or active biomonitoring, *i.e. in situ* caging of sentinels species (Beeby, 2001), organisms can be exposed to multiple sources of contamination like soil, vegetation, air… Thus, knowing the influence of exposure sources on metal accumulation by the bioindicator is necessary to accurately characterize the metal bioavailability to organisms (Fairbrother et al., 2007; Burger, 2008).

Among the soil fauna, the land snail *Cantareus aspersus* has been used for biomonitoring using snails caged in microcosms (Scheifler et al., 2003a; Gimbert et al., 2008a). Active biomonitoring allows all contamination sources to be taken into account (Scheifler et al., 2003a; Gimbert et al., 2008a)…). However, neither the contribution of each contamination sources on metal assimilation by snails and nor the influences of soil properties on this contribution have been studied. Indeed, previous studies on the contribution of contamination sources on metal accumulation (Scheifler et al., 2006; Fritsch et al., 2008) have shown that accumulated Cd came mainly from vegetation whereas the main contamination source of Pb is the soil but these studies did not consider the dynamic processes of bioavailability. In this purpose, active biomonitoring coupled with accumulation kinetics are relevant to evaluate the metal bioavailability to organisms via the modeling of assimilation fluxes (Gimbert et al., 2008a). After assimilation, snails are able to sequester metals in various compartments (Gimbert et al., 2008c). Even if sequestration and elimination of metals have been studied (Vijver et al., 2005; Notten et al., 2006; Gimbert et al., 2008c), the influence of the sources of the metals and soil parameters on excretion is still unknown.

Therefore, the present study aimed, based on an experimental design using spiked and artificially modified soil (i) to assess the relative contribution of soil and vegetation on the Cd and Pb assimilation and influence of these sources on the metal excretion by snails and (ii) to evaluate the influence of pH and OM on this contribution by using kinetic modeling of accumulation.

II. Material and method

II.1. Animals

Juvenile brown garden snails (*C. aspersus aspersus* Müller, 1774) were reared under controlled conditions as described previously (Gomot-de Vaufleury, 2000; ISO 15952, 2006). The individuals used for the test were subadults, reared for 7-9 weeks and weighing 5.11±0.75 g (±SD; n = 415) at the beginning of the experiment.

II.2. Exposition sources

II.2.a. Soil

An uncontaminated agricultural field soil (Chambornay-les-Pin, Eastern France) was used for further soil parameter modifications (Table 16). It was a sandy loam taken from the top layer (depth, 0–15

cm) of a maize field and transferred to the laboratory to be air-dried and, then sieved through a 4 mm mesh.

Soil characteristics (Table 16) were artificially modified by adding: (i) dried ground peat (Organisation for Economic Co-operation and Development (OECD), 1984) to raise organic matter content (soil P) from 1.4 to 8%; and/or (ii) $CaCO_3$ powder to obtain two nominal classes of pH (5 and 7) (Organisation for Economic Co-operation and Development (OECD), 1984; Van Gestel and Koolhaas, 2004).

The soils were spiked by spraying Cd ($CdCl_2$, 99.9% purity, Aldrich Chemical) and Pb ($PbSO_4$, 99.9% purity, Aldrich Chemical) in aqueous solution to reach nominal concentrations of 20 and 2000 $mg.kg^{-1}$ soil DW (Dry Weight), respectively. Deionised water was added to reach 50% of the water holding capacities of the different substrates. The soils were left to stabilise in the dark for one month (Pauget et al., 2011) before snail exposure.

Table 16: Main parameters of contamination sources (soils and lettuce)

Source		[Cd]	[Pb]	pH_w	OM	Clay	CEC	$[Al]_{ox}$	$[Fe]_{ox}$	Silts	Sands
		mg/kg	mg/kg	-	g/kg	g/kg	cmol+/kg	g/100g	g/100g	g/kg	g/kg
Contaminated soil	7C	18.0	1980	7.29	14.8	140	5.8	0.132	0.909	397	463
	7P	17.9	1980	7.35	74.5	166	11.9	0.133	0.884	430	404
	5P	18.1	2030	4.73	80.9	103	13.1	0.14	0.864	482	415
Uncontaminated soil	7C	0.263	33.1	7.7	15	132	5.75	0.133	0.907	376	492
	7P	0.21	25.6	7.6	76.4	150	13.2	0.127	0.849	406	444
	5P	0.221	27.2	4.93	77.7	163	12.3	0.135	0.867	416	421
Contaminated Lettuce	7C	72.7±15.2	144±118								
	7P	83.7±21.6	64.6±32.0								
	5P	113±2.57	80.4±63.3								
Uncontaminated Lettuce	7C	2.28±0.337	6.7±2.71								
	7P	2.43±0.748	2.00±1.23								
	5P	2.19±0.088	3.61±0.131								

II.2.b. Lettuce

During the experiment, snails were fed with lettuce having grown on the soil on which snails are exposed during two months. So, contaminated lettuce was obtained for each contaminated soil for better environmental relevance.

II.3. Experimental design

Accumulation kinetics have been determined on the basis of a 10-days experiment, in which snails were exposed to (i) contaminated soil with contaminated lettuce (S*L*), (ii) contaminated soil with uncontaminated lettuce (S*L), (iii) uncontaminated soil with contaminated lettuce (SL*), (iv) contaminated soil without lettuce (S*) and (v) contaminated lettuce without soil (L*).

For each treatment, ten snails were housed in each of three replicate polystyrene containers (24 x 21 x 8 cm) (ref. EIDBBAC001, Charles River IFFA-CREDO, L'Arbresle, France) containing a 1-cm layer (100 g dry mass) of soil prepared as previously described (except for the treatment L* were no substrate was added). The photoperiod was 18L/6D and the temperature was 20±2°C. The relative humidity was kept at 80 to 95%. Soil moisture content was maintained at its initial level by regular spraying

with demineralised water. Three times a week, the containers were cleaned, any remaining food was renewed and demineralised water was sprayed to prevent drying out of soil.
After 2, 4, 6, 8 and 10 days of exposure, one snail per treatment was randomly sampled. After the 10 days of exposure, organisms were transferred on the corresponding uncontaminated soil and fed with uncontaminated lettuce to assess the metal depuration and one snail per treatment was randomly sampled after 2, 5, 7 and 10 day of depuration.

II.4. Analytical procedures

II.4.a. Soil

Total Cd and Pb measurements were made by inductively-coupled plasma atomic emission spectrophotometry (ICP-AES) after digestion of the soil samples (250 mg) with hydrofluoric and perchloric acid and as described in (AFNOR, 1996). Analysis were performed by the *Laboratoire d'analyse des sols* of Arras (France), which benefits from the COFRAC (French accreditation committee) accreditation n°1-1380 for its analytical quality for metal measurements in soils. Soil characteristics were assessed/described by the same laboratory.

II.4.b. Lettuce

Lettuce was freeze-dried till constant weight (0.08±0.07 mg) and digested in a solution of 6-mL-nitric acid and 2-mL-hydrogen peroxide (HNO_3 65%, H_2O_2 30%, Carlo-Erba analytical quality). After digestion, samples were diluted adding ultra-pure water (18.2 MΩ/cm^2) as previously described by Fritch et al. (Fritsch et al., 2011) and analyzed by ICP OES (iCAP 6000, Thermo Fisher Scientific). The validity of the analytical methods used was checked by analysing standard biological reference material (ICHTJ-cta-VTL-2, Institute of Nuclear Chemistry and Technology, Poland).

II.4.c. Snails

Snails for analysis were placed in clean containers and fasted for 48h (the faeces were removed after 24h) then weighed. The snails were sacrificed by freezing at -80°C. After thawing, the whole soft body was removed from the shell and the foot and the viscera were separated (Gomot-de Vaufleury and Pihan, 2002). Only viscera were studied in this work because they contain the digestive gland (hepatopancreas) which is the main site of metal accumulation and storage in snails (Hopkin S. P., 1989). The viscera were oven-dried at 60°C till constant weight (0.228±0.049 g, n=415) and digested in nitric acid (HNO_3 65%, Carlo-Erba analytical quality) as previously described (Gomot-de Vaufleury and Pihan, 2002). After digestion, samples were diluted adding ultra-pure water (18.2 MΩ/cm^2), filtered in an ash free filter paper and analysed by ICP OES (iCAP 6000, Thermo Fisher Scientific). The validity of the analytical methods used was checked by analysing standard biological reference material (TORT-2, lobster hepatopancreas; National Research Council of Canada–Institute for National Measurement Standard, Ottawa, ON, Canada).

II.5. Statistical analyses

II.5.a. Accumulation modeling

Bioavailability results from a dynamic interaction between the metal concentration in the soil (environmental availability) and the physiology of the target species. For its assessment, a one-compartment model was used to fit the accumulation kinetic data (Gimbert et al., 2006; Pauget et

al., 2011). This model expresses the dynamic change of metal concentration in the snail viscera (C_{sn} in $mg_{metal}.kg\ DW_{sn}^{-1}$) over time, following the equation (Eq. 1):

(1) $$C_{sn}(t) = C_{sn}(0) + \frac{a}{k_2}\ (1 - e^{-k_2 t})$$

where *a* is the assimilation flux constant ($mg_{metal}.kg\ DW_{sn}^{-1}.d^{-1}$) considered as an indicator of the metal bioavailability to snails (Gimbert et al., 2008a; Pauget et al., 2011); k_2 is the excretion rate constant (d^{-1}); and t is time (days). $C_{sn}(0)$ is the average metal concentration measured in ten snails at the beginning of the experiment ($mg_{metal}.kg\ DW_{sn}^{-1}$).

When negative value estimates of excretion rate k_2 (which have not biological sense) were modelled by Eq.1, for an accurate assessment of the assimilation flux (a) in these particular cases, a linear model was substituted to Eq.1 according to (Eq. 2):

(2) $$C_{sn}(t) = C_{sn}(0) + a * t$$

II.5.b. Depuration modeling

During the depuration phase, excretion can occur and lead to variations of metal concentrations in snails (C_{sn}) with time according to the equation (Eq. 3) (Gimbert et al., 2006):

(3) $$C_{sn}(t) = C_{sn}(10) - C_{sn}(10) * (1 - e^{-k_{2d}(t - t_c)})$$

where $C_{sn}(10)$ is the metal concentration at the end of the exposure period ($mg.kg\ DW_{sn}^{-1}$) ; k_{2d} is the excretion of metal (d^{-1}); t_c is the time (days) at which animals were transferred to the clean soil and t is the time (days) since the beginning of the experiment. All negative estimates of excretion (k_{2d}) were considered as not biologically relevant and therefore not presented in the tables of results.

The accumulation and excretion parameters were estimated by fitting the models with a mixed-effects procedure (non linear mixed-effect (nlme) or linear mixed-effect (lme), (Lindstrom and Bates, 1990) allowing for nested random effects. The within-group errors were allowed to be correlated and/or have unequal variances. The nlme integrated the soil as a fixed factor and the container as a random effect. The lme integrated the metal concentration in snail as dependent variable and the soil as explanatory variable. For all models (lme and nlme), when residuals were skewed, variance functions (power and exponential) were applied and the best model is selected according to Akaike's Information Criterion (AIC, pgirmess package) (Burnham and Anderson, 2004). Statistical differences in parameter estimates between treatments were judged from the absence of overlap of their 95% confidence intervals (95% CI). All statistical analyses were performed with the free statistics software package R (version 2.10.1, (R Development Core Team, 2011).

II.6. Determination of the contribution of soil and lettuce in the Cd and Pb bioavailability

Firstly, the additivity of the assimilation fluxes is checked. We consider that the sum of $a_{S*}+a_{L*}$ or $a_{S*L}+a_{SL*}$ is not different of a_{S*L*} when the relative standard deviation (RSD) is inferior to 15%.

When the additivity has been validate, the relative contribution of soil and lettuce in the metal bioavailability to snails is quantified by the the mean of the percentage of assimilation flux of snails exposed to one contamination source to the assimilation fluxes of snails exposed to both contamination sources according to:

$$Contribution\ of\ soil = 0.5 * (\frac{a_{S*L}}{a_{S*L*}} * 100 + \frac{a_{S*}}{a_{S*L*}} * 100)$$

$$Contribution\ of\ lettuce = 0.5 * (\frac{a_{SL*}}{a_{S*L*}} * 100 + \frac{a_{L*}}{a_{S*L*}} * 100)$$

If additivity is not observed, the contribution of soil and lettuce is determined by remplacing a_{S*L*} by the sum of $a_{S*L}+a_{SL*}$ or $a_{S*}+a_{L*}$.

III. Results

III.1. Accumulation and depuration kinetics

At the beginning of the exposure, the internal concentrations in snails were 0.73 ± 0.10 mg kg^{-1} for Cd and 0.59 ± 0.26 mg kg^{-1} for Pb (n = 10). After 10 days of exposure, only 0.9% (n=2) snails have died showing the absence of letality of treatment. Cd and Pb have been accumulated by snails as testified by the significant assimilation fluxes ranking from 0.604 to 4.34 mg Cd kg_{sn}^{-1} d^{-1} and from 0.954 to 66.7 mg Pb kg_{sn}^{-1} d^{-1} (for Cd and Pb, respectively). Only for the modalities 7C SL* and 7P S* (p-values = 0.106 and 0.267) for Cd and 7P SL* (p-value = 0.169) for Pb positive but no significant assimilation fluxes are have been modeled (Table 17). Overall the highest assimilation fluxes values for Cd were observed when the contaminated lettuce was offered, whereas for Pb, snails exposure to the contaminated soil lead to the highest assimilation fluxes.

During the exposure phase, for Cd, no significant (*p-value*>0.05) excretion rate (k_2) have been modeled on the exposure duration. For Pb, few excretion rates have been modeled. Only one excretion rate was significant on the modality 5P S*L*.
During the depuration phase, the Cd seems to be not excreted by snails. Only three positive values of k_{2d} have been modeled (two are significant and one is no significant) for the Cd (Table 17). Contrarily to Cd, the Pb seems to be excreted as shown by the ten modeled positive k_{2d} (4 significant and 6 no significant).

III.2. Influence of soil properties on metal bioavailability to snails

III.2.a. Influence of organic matter content

An increase of organic matter content leads to a decrease of Cd bioavailability to snails when they are exposed to the modalities presenting contaminated lettuce (L*) decreasing from 3.93 to 2.94 mgCd kg_{sn}^{-1} d^{-1} (S*L*), from 3.15 to 1.36 mgCd kg_{sn}^{-1} d^{-1} (SL*) and from 3.33 to 1.68 mgCd kg_{sn}^{-1} d^{-1} (L*). In the modalities with contaminated soil (S*), a slight increase is observed (Table 17 and Figure 36). For Pb, an increase of OM content leads to an increase of its bioavailability to snails on the modalities S*L*, S*L and S* as showed by the assimilation fluxes increasing from 19.5 to 23.8 mgPb kg_{sn}^{-1} d^{-1} (S*L*), from 16.3 to 21.3 mgPb kg_{sn}^{-1} d^{-1} (S*L) and from 7.3 to 20.4 mgPb kg_{sn}^{-1} d^{-1} (S*) (Table 17 and Figure 37).

Table 17: Estimates of kinetic parameters for Cd and Pb accumulation and depuration in *Cantareus aspersus* exposed to the contaminated soils and/or lettuce. (a): assimilation flux reflecting bioavailability, k_2:excretion rate during exposure time, k_{2d}: excretion rate during depuration time, $C_{sn}(10)$: modeled snails viscera concentration after 10 days of exposure, $C_{sn}(20)$: modeled snails viscera concentration after 10 days of exposure and 10 days of depuration.

	ACCUMULATION							DEPURATION			
Modality	a	95IC	*p-value*	k_2	95IC	*p-value*	$C_{sn}(10)$ mod	k_{2d}	95IC	*p-value*	$C_{sn}(20)$ mod
	$mg_{metal}\ kg_{sn}^{-1}\ d^{-1}$			d^{-1}			$mg_{metal}\ kg_{sn}^{-1}$	d^{-1}			$mg_{metal}\ kg_{sn}^{-1}$
Cadmium											
7C S*L*	3.93	3.41/4.44	<0.001	0.216	0.102/0.330	0.111	16.8				
7C S*L	0.736	0.659/0.813	<0.001				8.09				
7C SL*	3.15	2.21/4.09	0.011	0.170	0.071/0.269	0.145	15.9				
7C S*	0.604	0.465/0.743	0.002	0.005	-0.008/0.019	0.721	6.62	0.046	0.038/0.054	<0.001	4.18
7C L*	3.33	2.17/4.49	0.024	0.209	0.084/0.334	0.155	14.7				
7P S*L*	2.94	2.74/3.14	<0.001				30.1	0.052	0.038/0.066	0.007	17.9
7P S*L	1.07	0.794/1.35	0.046	0.181	-0.078/0.440	0.536	5.67				
7P SL*	1.36	0.981/1.74	0.01	0.028	-0.074/0.130	0.809	12.6				
7P S*	1.54	0.338/2.74	0.267	0.300	-0.033/0.634	0.429	5.60	0.017	0.008/0.026	0.113	4.73
7P L*	1.68	1.44/1.92	<0.001				17.5				
5P S*L*	4.34	3.85/4.83	<0.001	0.180	0.083/0.277	0.118	20.8				
5P S*L	1.95	1.52/2.38	0.002	0.074	-0.127/0.275	0.744	14.5				
5P SL*	2.83	1.92/3.73	0.015	0.326	0.161/0.490	0.098	9.07				
5P S*	1.19	0.993/1.39	<0.001				12.6				
5P L*	3.69	2.76/4.62	0.041	0.288	0.058/0.518	0.277	12.8				
Lead											
7C S*L*	19.6	14.2/24.8	0.006	0.051	-0.098/0.201	0.76	154	0.002	-0.018/0.023	0.913	151
7C S*L	16.3	13.9/18.8	<0.001				164	0.046	0.029/0.064	0.034	103
7C SL*	0.954	0.895/1.01	<0.001				10.1				
7C S*	7.31	6.07/8.54	<0.001				73.7	0.047	0.029/0.064	0.039	46.1
7C L*	1.33	1.13/1.54	<0.001				13.9				
7P S*L*	23.8	20.8/26.9	<0.001				239	0.074	0.052/0.096	0.013	114
7P S*L	21.3	19.3/23.4	<0.001				214	0.031	0.008/0.055	0.251	157
7P SL*	1.35	0.513/2.19	0.169	0.298	0.034/0.561	0.325	4.89				
7P S*	20.4	13.5/27.2	0.021	0.230	0.033/0.427	0.31	80.4				
7P L*	0.579	0.494/0.664	<0.001				6.38	0.038	0.021/0.056	0.083	4.36
5P S*L*	66.7	53.3/80.2	0.001	0.316	0.202/0.430	0.028	203				
5P S*L	22.5	20.8/24.3	<0.001				226	0.011	-0.002/0.024	0.438	202
5P SL*	3.81	3.21/4.40	<0.001				38.7	0.056	0.040/0.072	0.013	22.1
5P S*	20.6	19.3/21.9	<0.001				206	0.035	0.005/0.065	0.306	146
5P L*	4.15	2.89/5.40	0.009				42.1	0.050	0.028/0.074	0.071	25.2

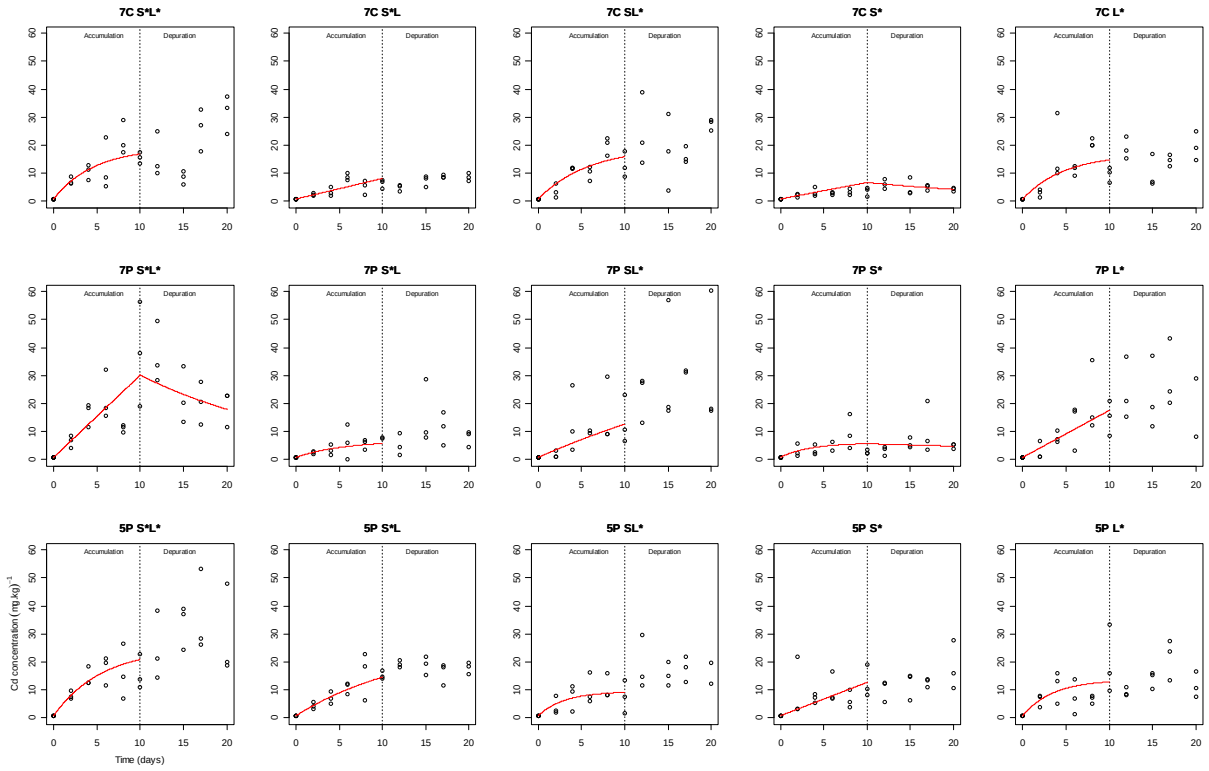

Figure 36: Modeled accumulation kinetics of Cd in *C. aspersus* snails exposed to soils C and soils P (i.e. soil C + peat) with decreasing pH. The Cd concentrations in snail's viscera are expressed on a dry weight basis. The curve represents the accumulation kinetic. Each data point represents an individual snail.

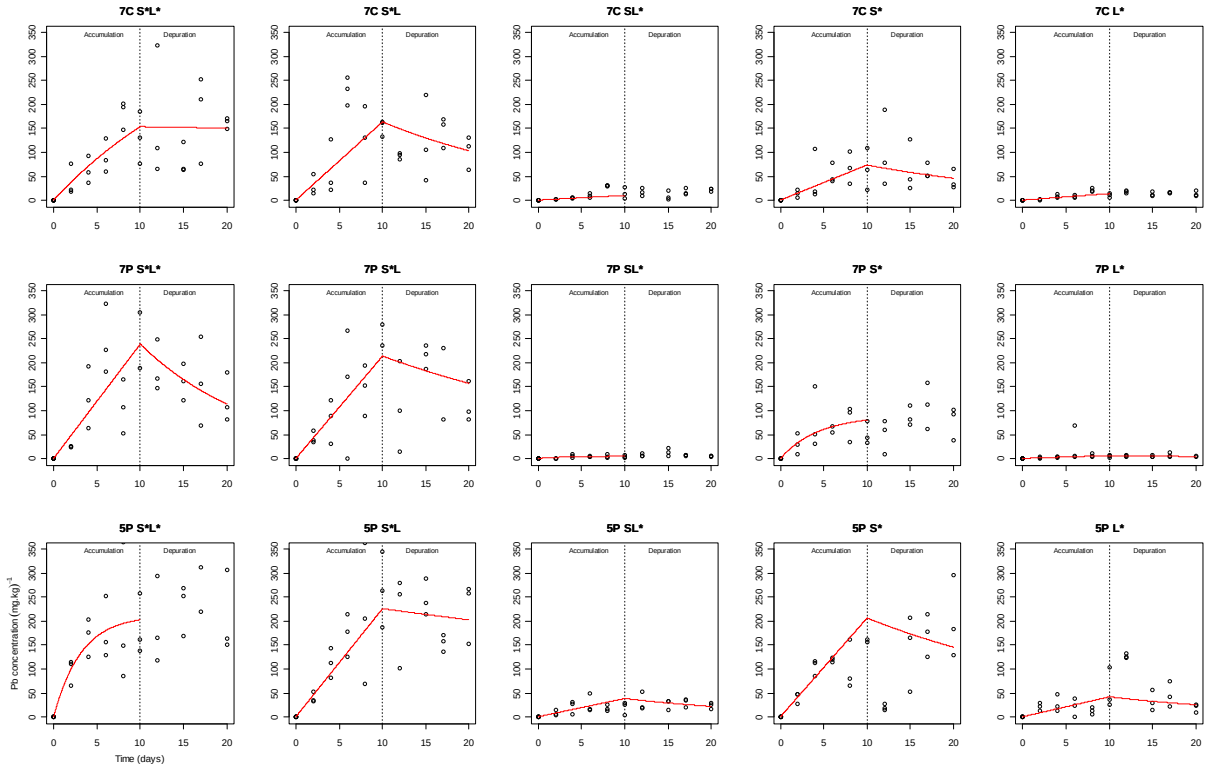

Figure 37: Modeled accumulation kinetics of Pb in *C. aspersus* snails exposed to soils C and soils P (i.e. soil C + peat) with decreasing pH. The Cd concentrations in snail's viscera are expressed on a dry weight basis. The curve represents the accumulation kinetic. Each data point represents an individual snail.

III.2.b. Influence of pH

A decrease of 2.6 units of pH leads to an increase of Cd bioavailability in all modalities going from 2.94 to 4.34 $mg_{Cd}\ kg_{sn}^{-1}\ d^{-1}$ (S*L*), from 1.07 to 1.95 $mg_{Cd}\ kg_{sn}^{-1}\ d^{-1}$ (S*L), from 1.36 to 2.83 $mg_{Cd}\ kg_{sn}^{-1}\ d^{-1}$ (SL*) and from 1.68 to 3.69 $mg_{Cd}\ kg_{sn}^{-1}\ d^{-1}$ (L*). For Pb, a significant increase of bioavailability is observed on the modality S*L* at the lower pH with assimilation going from 23.8 to 66.8 $mg_{Pb}\ kg_{sn}^{-1}\ d^{-1}$ and on modality L* with assimilation going from 0.579 to 4.15 $mg_{Pb}\ kg_{sn}^{-1}\ d^{-1}$, but no difference is observed between the modalities S*L and S*.

III.3. Determination of the contribution of soil and plant to metal bioavailability to snails

Firstly we want to determine whether the sum of the assimilation fluxes of snails exposed to (i) soil and (ii) lettuce correspond to the assimilation flux of snails exposed to the both sources. For Cd, the sums of assimilation fluxes ($a_{S*L}+a_{SL*}$ and $a_{S*}+a_{L*}$) correspond to the measured assimilation fluxes on modalities S*L* for the three soils (Table 18). For Pb, the sum of the assimilation fluxes of the two sources corresponds to the bioavailability to snails of both sources on soil 7P and on soil 7C with the modalities S*L and SL* and on soil 7P with the modalities S* and L*. For the other cases, the assimilation flux with both contamination sources is higher than the sum of the assimilation fluxes with one contamination sources. However, on the soil 5P, the Pb bioavailability to snails exposed to the two contamination sources may be overestimated, indeed the sum of $a_{S*L}+a_{SL*}$ and $a_{S*}+a_{L*}$ are the same and the internal concentration in Pb after 10 days of exposure are quite similar between the modality S*L*, S*L and S*.

Table 18: Comparison between assimilation fluxes modeled when snails are exposed to the two contamination sources and the sum of assimilation fluxes of snails exposed only to one contamination source. %RSD : % of relative standard deviation (compared to the modality a_{S*L*}). %RSD in italic are higher than the limit (15%)

Soil	parameter	Metal			
		Cd (mg $kg^{-1}\ d^{-1}$)	%RSD	Pb (mg $kg^{-1}\ d^{-1}$)	%RSD
7C	a_{S*L*}	3.93		19.5	
	$a_{S*L}+a_{SL*}$	3.88	1.12	17.3	11.7
	$a_{S*}+a_{L*}$	3.93	0.102	8.64	*55.8*
7P	a_{S*L*}	2.94		23.8	
	$a_{S*L}+a_{SL*}$	2.43	*17.2*	22.7	4.83
	$a_{S*}+a_{L*}$	3.22	9.46	21	12.0
5P	a_{S*L*}	4.34		66.8	
	$a_{S*L}+a_{SL*}$	4.78	10.2	26.4	*60.5*
	$a_{S*}+a_{L*}$	4.88	12.5	24.7	*62.9*

The relative contribution of each contamination sources is presented in the Table 19. Without taking into account the soil parameters influence, the contribution of soil to Pb assimilation by snails is about three times higher than for Cd (85% against 35%, respectively) whereas contribution of lettuce is more than six time higher for Cd than for Pb (72% against 8%, respectively). When taking into account the influence of soil characteristics, we highlight that raising the OM content increases the soil contribution of 35% for Cd and of 4% for Pb, thereby decreasing the lettuce contribution. A decrease of two pH units decreases the soil contribution of 16% for Cd and 3% for Pb, thereby increasing the lettuce contribution.

Table 19: Contribution of soil and lettuce to metal bioavailability to snails

Contribution	Soil	Metal	
		Cd (%)	Pb (%)
Soil	7C	17.0	83.4[b]
	7P	52.3[a]	87.5
	5P	36.2	84.4[c]
	Mean	35.2	85.1
Lettuce	7C	82.4	4.88[b]
	7P	57.1[a]	4.04
	5P	75.1	15.6[c]
	Mean	71.6	8.17

a: calculated by using only mono source exposure,

b: calculated by using only both source exposure

c: calculated by remplacing $a_{S^*L^*}$ by the sum of $a_{S^*L}+a_{SL^*}$ or $a_{S^*}+a_{L^*}$.

IV. Discussion

IV.1. Soil and plant contributions to metal bioavailability to snails

In this study we characterized bioavailability to snails of Cd and Pb present in soil and lettuce by modeling assimilation fluxes. Our experimental design has permitted to assess the contribution of both soil and lettuce on metal bioavailability to snail. Moreover, the use of accumulation kinetics allows the assessment of the influence of soil parameter like OM content and pH on metal fluxes in organisms (assimilation and excretion) whereas these influences are not highlighted when comparing the concentration of metal in snail viscera after 10 days of exposure.

We evidenced different influences of contamination sources on the amount and fluxes of metal assimilated by snails. Indeed, results showed that Cd is mainly assimilated from lettuce (68.1%) whereas assimilated Pb came mainly from soil (89.9%). These results are in accordance with a previous work based on two durations of exposure which estimated that the soil contribution to metal bioaccumulation by snails can be higher than 80% for Pb and from 2 to 40% for Cd depending on the stage of development of the plant (Scheifler et al., 2006). The use of kinetics studies to identify the contamination sources allows the determination of the fluxes of metal in organisms of each source and permits to highlight difference even if same concentrations in metal in viscera at the end of exposure are measured. The determination of the fluxes, the sources and speciation of metal assimilated are important during bioavailability assessment because they condition the adverse effects of metals (Notten et al., 2005; Peijnenburg et al., 2007; Calhôa et al., 2011). The difference between the Cd and Pb in the plant contribution can be due to the difference of metal localization in plant. Indeed, Cd may be mainly present as free metal ion in leaves (Leita et al., 1991; Mendoza-Cozatl et al., 2011) and thus may be easily bioavailable to snails whereas Pb is located in the cell walls (Qureshi et al., 1986). The different sequestration of metal in plant is important to take into account. For instance, it has been shown that Cd bind to metallothioneins is less bioavailable to isopod than Cd bind to heat denatured proteins (Monteiro et al., 2008).

The similar value of assimilation fluxes of snails exposed to both contamination sources and the addition of assimilation fluxes of snails exposed only to one contamination source (S*+L* or S*L+SL*) suggest that the feeding behavior of snails is not modified by the presence of metal in food (soil or plant) as previously observed by (Noret et al., 2005; Sinnett et al., 2009). When we focus on soil parameter influence on the contribution of the sources on metal bioavailability to snails, we observe an influence of OM content. Indeed, the addition of organic matter increases the contribution of soil for the Cd and Pb bioavailability, probably due to the increase of metals mobility in soil in relation to the increase of the OM content in the soil solution (Girard et al., 2005). Another hypothesis is an increase of soil ingestion by snails. When deriving uptake rates (k_1= a/metal concentration in soil or in lettuce, representing the exposure, (Pauget et al., 2011)) on the modalities S*L with the soil 7C and 7P, we observe an increase of soil exposure showing the increase of soil ingestion. Snails have to eat soil to pick up nutrients (Gomot et al., 1989), the addition of OM in the soil 7P may decrease the concentration of nutrients in soil and may lead to force snails to eat more soil to satisfy their physiological needs. Moreover, a soil with a high OM content may be more palatable, the OM being consumed by snails to their growth (Elmslie, 1998).

When focusing on the influence of a decrease of pH, we can observe an increase of the contribution of the lettuce on Cd and Pb assimilation. This increase may be due to the increase of its metal concentration leading to the increase of assimilation fluxes of snails exposed only to lettuce. Even if the soil contribution has decreased, assimilation fluxes from soil have been increased but in a lesser extent than the increase of assimilation fluxes of snail exposed to lettuce. This let suppose difference between the bioavailability of metal coming from soil and plants. An increase of Cd and Pb bioavailability has already been shown with a 2-pH-units decrease by Pauget et al. (Pauget et al., 2011) with soil as unique source of contamination. It is mainly due to the modification of metal mobility in soil as observed in previous studies (Sterckeman et al., 2004; Van Gestel and Koolhaas, 2004) which have shown that Cd and Pb speciation depended partly on soil acidity. Indeed acidic soils might modify colloid effects on proton activity, increasing their solubility and their bioavailability to snails and lettuce. Moreover, Sauvé et al. (Sauvé et al., 2000) reported that the pH explained 47% of Pb and Cd partitioning coefficients.

IV.2. Accumulation and excretion of metals

Even if classical exposure studies work on 28-days duration, the 10-days exposure used in this study has allowed the determination of the metal bioavailability to snails for both contamination sources. However, this time is too short to clearly identify if snails have excreted metal even if a tendency is observed. Indeed, although excretion rate are not significant, the Pb may be more retained in the snails tissues compared to Cd during the exposure phase whereas Cd was not excreted during the depuration phase. The depuration period allows the identification of positive value of excretion of Cd in only three modalities and of Pb in eight modalities. For both metal, neither the soil properties nor the contamination source influence the excretion rates. However, even if differences in Cd and Pb excretions by snails have been demonstrated (long term storage of Cd and excretion of Pb) (Hopkin, 1989; Spurgeon and Hopkin, 1999; Dallinger et al., 2004a), we cannot identify these differences in our study with such a short time of exposition and depuration. Moreover, during this study the metal concentration in foot has not been measured. The absence of clear Cd and Pb excretion during the depuration phase may be due to a metal transfer from foot to viscera. Indeed, in the absence of external metal source during the depuration phase, only the foot can be involved, the shell being not a sink for metal (Laskowski and Hopkin, 1996). The dermal route via the foot being a significant route

for metal assimilation (Coeurdassier et al., 2002) it is possible that an income of Cd and Pb appears coming from foot during the depuration phase or/and that snails are able to relocate metal in different part of its body (Gimbert et al., 2006) leading to the hidden of the metal depuration by a metal flux coming from foot.

V. Conclusion

This study brings new information necessary to better understand the metal bioavailability and transfer to snails. Indeed, the consideration of contamination sources and respective influence of environmental parameters such as OM, pH on both phyto- and zooavailability is necessary to understand metal transfer during *in situ* metal bioavailability assessment, organisms being exposed to multiple contamination sources. Indeed, the great contribution of lettuce in Cd assimilation by snails reinforces the fact that only measuring the total soil concentration is not relevant to assess Cd bioavailability to snails even if as observed by Pauget et al. (Pauget et al., 2012), snails are able to assimilate Cd coming from the strongly bound fraction. These data on sources contribution will improve the bioavailability modeling under a complex exposure (*e.g.* passive biomonitoring) and the improvement of biodynamic model to assess metal bioavailability to snails for risk assessment purpose.

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Chapitre 2 : Soil parameters are key factors to predict metal bioavailability to snails based on chemical extractant data.

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Soil parameters are key factors to predict metal bioavailability to snails based on chemical extractant data

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ABSTRACT

Although soil characteristics modulate metal mobility and bioavailability to organisms, they are often ignored in the risk assessment of metal transfer. This paper aims to determine the ability of chemical methods to assess and predict cadmium (Cd), lead (Pb) and zinc (Zn) environmental bioavailability to the land snail *Cantareus aspersus*. Snails were exposed in the laboratory for 28 days to 17 soils from around a former smelter. The soils were selected for their range of pH, organic matter, clay content, and Cd, Pb and Zn concentrations. The influence of soil properties on environmental availability (estimated using HF-$HClO_4$, EDTA, $CaCl_2$, NH_4NO_3, $NaNO_3$, free ion activity and total dissolved metal concentration in soil solution) and on environmental bioavailability (modelled using accumulation kinetics) was identified. Among the seven chemical methods, only the EDTA and the total soil concentration can be used to assess Cd and Pb environmental bioavailability to snails ($r^2_{adj} = 0.67$ and 0.77, respectively). For Zn, none of the chemical methods were suitable. Taking into account the influence of the soil characteristics (pH and CEC) allows a better prediction of Cd and Pb environmental bioavailability ($r^2_{adj} = 0.82$ and 0.83, respectively). Even though alone none of the chemical methods tested could assess Zn environmental bioavailability to snails, the addition of pH, iron and aluminium oxides allowed the variation of assimilation fluxes to be predicted. A conceptual and practical method to use soil characteristics for risk assessment is proposed based on these results. We conclude that as yet there is no universal chemical method to predict metal environmental bioavailability to snails, and that the soil factors having the greatest impact depend on the metal considered.

Résumé :

Bien que les caractéristiques du sol modulent la mobilité des métaux et leur biodisponibilité pour les organismes, elles sont souvent ignorées lors d'études d'évaluation des risques. Cette étude vise à déterminer la capacité des méthodes chimiques à évaluer et à prédire la biodisponibilité environnementale du cadmium (Cd), du plomb (Pb) et du zinc (Zn) aux escargots *Cantareus aspersus*. Les escargots ont été exposés en conditions contrôlées pendant 28 jours à 17 sols provenant des alentours d'une ancienne fonderie. Ces sols ont été sélectionnés pour leur gamme de pH, de matière organique, de teneur en argile, et pour leurs concentrations en Cd, Pb et Zn. L'influence des propriétés du sol sur la disponibilité environnementale (estimée à l'aide HF-$HClO_4$, EDTA, $CaCl_2$, NH_4NO_3, $NaNO_3$, l'activité ionique et la concentration totale de métal dissous dans la solution du sol) et sur la biodisponibilité environnementale (modélisée grâce aux cinétiques d'accumulation) a été identifiée. Parmi les sept méthodes chimiques utilisées, seules une extraction à l'EDTA et la concentration totale du sol peuvent être utilisées pour évaluer la biodisponibilité environnementale du Cd et du Pb (r^2_{adj} = 0,67 et 0,77, respectivement). Pour le Zn, aucune des méthodes chimiques ne sont utilisables. La prise en compte de l'influence des caractéristiques du sol (pH et CEC) permet une meilleure prédiction de la biodisponibilité environnementale du Cd et du Pb (r^2_{adj} = 0,82 et 0,83, respectivement). Même si aucune des méthodes chimiques testées seules ne peut évaluer la

biodisponibilité environnementale du Zn aux escargots, l'ajout du pH, des oxydes de fer et d'aluminium permet d'améliorer la prédiction des flux d'assimilation du Zn. Une méthodologie conceptuelle pour identifier une méthode chimique permettant d'estimer la biodisponibilité des métaux est proposée sur la base de ces résultats. Nous concluons qu'à l'heure actuelle, il n'existe pas de méthode chimique universelle pour prédire la biodisponibilité des métaux de l'environnement aux escargots, et que les facteurs du sol ayant le plus d'influence sur la biodisponibilité varient en fonction du métal considéré.

I. Introduction

Among contaminants, metals are considered to be one of the main threats concerning soil quality degradation (Commission of the European communities, 2006). Indeed, metals cause environmental disturbances for both organisms and habitats by direct or indirect toxicity (Moriarty, 1999) and may affect the animal and human populations (Huff et al., 2007; Qingdong et al., 2007). Currently, risk assessment procedures often ignore the soil characteristics, though they may influence metal mobility in soil (Young et al., 2000) and their transfer to primary producers and primary consumers (Kabata-Pendias, 2004). Transfer corresponds to the passage of a pollutant from a compartment (biotic or abiotic) to organisms and is conditioned by both exposure and bioavailability of metals. Bioavailability is the part of the total pollutant in soil which is available or made available in a dynamic manner over time to an organism from its direct environment (Peijnenburg and Jager, 2003a). Bioavailability can be divided into three different concepts (Lanno et al., 2004; ISO 17402, 2008): the environmental availability (EA), is governed by the physico-chemical processes that regulate metal partition between the solid and liquid phases of soil. The environmental bioavailability (EB) corresponds to the physiological processes which control assimilation of the pollutant by the organism. The toxicological bioavailability corresponds to the part of pollutant that reaches and interacts with the sites of toxic action. Nowadays, biological approaches are recommended to assess metal bioavailability (ISO 17402, 2008). Indeed, many organisms living on or in soil are known to give an indication of soil quality and contamination (van Straalen et al., 2008b). Among these organisms, the garden snail (*Cantareus aspersus*) is a biological indicator which integrates three contamination routes: respiratory (from atmospheric source), digestive (from plant and soil sources) (Gomot et al., 1989) and dermal routes (diffusion of metal through the foot epithelium) (Coeurdassier et al., 2002) and was previously used to characterize metal EB (Gimbert et al., 2006; Pauget et al., 2011).

In order to improve soil quality and risk assessment, our aim here was to determine the ability of seven chemical methods to assess and predict metal EB to snail. As bioavailability is influenced by soil metal contamination, by the physico-chemical properties of the soil and by the physiology of the organism in question, the chemical method has to mimic all these aspects. Thus, it is now a priority to find a chemical method able to assess and predict metal EB to the snail, knowing how soil properties influence both EA and EB. EA (C_x: concentration assumed to be available) was estimated here by means of seven chemical extracts. EB of cadmium (Cd), lead (Pb) and zinc (Zn) was estimated by means of their accumulation kinetics and calculation of their assimilation flux (a). Indeed, the assimilation flux corresponds to the flux of metal crossing the biological membranes of the organism and integrates both the physico-chemical and physiological processes which govern metal assimilation. Then, a three-step approach was used: firstly the influence of soil characteristics on (i) EB and (ii) EA was expressed by mono- and multivariate regression. Secondly, the best chemical method assessing metal EB was selected on the basis of its ability to give an indication of the influence of soil characteristics either on bioavailable (a) or available metal concentrations (C_x). Finally, using the chemical extract selected and soil parameters, equations were developed to predict EB of Cd, Pb and Zn to snails.

II. Materials and methods

II.1. Animals

Garden snails (*Cantareus aspersus*) were bred as described by Gomot-de Vaufleury (Gomot-de Vaufleury, 2000) under controlled conditions. The individuals used for the test were subadults, reared for 7 to 9 weeks and weighing 5.0±0.6 g (n=516).

II.2. Soils

Seventeen soils were selected in the area impacted by the former smelter of Metaleurop (Nord-Pas-de-Calais, France, (Douay et al., 2009; Fritsch et al., 2011)) for their range in pH, organic matter (OM) and clay content and total contamination in Cd, Pb and Zn. Their characteristics are summarised in Table 20. The soils were sampled in the first 30 cm top layer after removal of vegetation residues. A mixed sample (from 3 sampling points) was prepared to free the tests from spatial heterogeneity of the contamination. Fresh soil aggregates were manually broken up, the coarse elements were removed and the soils were sieved through a 4-mm-mesh and air-dried (60°C). One month before the experiment, they were humidified to 50% of the water-holding capacity with demineralised water and maintained at constant temperature (20±2°C) in the dark to equilibrate.

Table 20: Physico-chemical characteristics of the 17 soils

Soil	pH_{water}	Clay ($g.kg^{-1}$)	Silt ($g.kg^{-1}$)	Sand ($g.kg^{-1}$)	OM ($g.kg^{-1}$)	CEC ($cmol.kg^{-1}$)	$[Al]_{ox}$ ($g.100g^{-1}$)	$[Fe]_{ox}$ ($g.100g^{-1}$)	$[Cd]_{tot}$ ($mg.kg^{-1}$)	$[Pb]_{tot}$ ($mg.kg^{-1}$)	$[Zn]_{tot}$ ($mg.kg^{-1}$)
A	7.7	294	575	131	56	19.3	0.129	0.762	6.4	683	1030
B	5.7	212	536	252	66	17.9	0.111	0.608	9.3	690	677
C	6.6	220	516	264	51	16.6	0.091	0.551	15.1	688	1280
D	7.3	121	514	365	118	12.9	0.101	0.998	23.1	1570	1560
E	8.1	227	576	197	56	15.8	0.110	0.758	15.7	671	854
F	7.7	242	566	192	85	19.6	0.110	0.889	32.1	1680	2050
G	7.6	168	437	395	58	11.6	0.147	1.140	38.6	1920	2830
H	5.5	257	484	259	145	26.4	0.142	1.130	22.4	2120	1080
I	6.8	180	351	469	245	20.7	0.196	1.570	28.1	2450	2850
J	6.2	192	435	373	90	17.5	0.131	0.717	13.1	938	870
K	7.4	106	244	650	349	19.1	0.098	1.260	8.1	524	660
L	8.2	151	344	505	132	12.2	0.115	5.880	13.5	2890	2780
M	8.1	201	483	316	204	16.4	0.101	1.230	11.0	2030	1930
N	8.1	66	206	728	221	9.2	0.145	4.860	80.8	37100	33700
X	7.9	240	496	126	61	15.2	0.151	0.560	17.4	911	1092
Y	7.0	278	584	138	28	17.9	0.100	0.626	6.8	392	430
Z	7.9	330	414	256	37	19.7	0.224	1.050	3.1	159	287
min	5.5	66	206	126	28	9.2	0.091	0.551	3.1	159	287
median	7.6	212	484	264	85	17.5	0.115	0.998	15.1	938	1092
max	8.2	330	584	728	349	26.4	0.224	5.880	80.8	37100	33700

II.3. Exposure modalities

For each soil, six snails were housed in five replicate polystyrene containers (24 x 21 x 8 cm) (ref. EIDBBAC001, Charles River IFFA-CREDO, L'Arbresle, France) containing a 1-cm layer (100 g dry weight) of each soil. The snails were fed *ad libitum* with uncontaminated (0.41±0.04 mgCd.kg^{-1} DW, 1.59±0.48 mgPb.kg^{-1} DW and 12.71±2.28 mgZn.kg^{-1} DW) pieces of fresh lettuce put in a Petri dish. Therefore, the only source of contamination was the soil by both dermal and digestive routes (Gomot et al., 1989; Coeurdassier et al., 2002). The photoperiod was 18L/6D and the temperature 20±2°C. The relative humidity was kept at 80 - 95%. Soil moisture content was maintained at its initial level by regular spraying with demineralised water. Three times a week, the containers were cleaned, and the food renewed.

After 0, 2, 5, 7, 14, 21 and 28 days of exposure to each soil, one snail was randomly sampled from each of the five replicate containers.

Sampled snails were placed in clean containers and fasted for 48h (the faeces were removed after 24h) and then weighed. The snails were sacrificed by freezing (at -80°C) before analysis. After thawing, the whole soft body was removed from the shell and the foot and the viscera separated (Gomot-de Vaufleury and Pihan, 2002). Only viscera were analysed in this work because they contain the digestive gland which is the main site of metal accumulation and storage in snails (Hopkin, 1989). The viscera were oven-dried at 60°C till constant weight (0.24±0.05 g, n= 516), digested in nitric acid (65% HNO_3, Carlo-Erba analytical quality) and analysed by graphite furnace atomic absorption spectrometry (GFAAS), as previously described (Gomot-de Vaufleury and Pihan, 2002). The validity of the analytical methods was checked by reference to standard biological material (TORT-2, lobster hepatopancreas; National Research Council of Canada–Institute for National Measurement Standard, Ottawa, ON, Canada). The average deviations from the certified values were 4.6±3.4% (*n*=10), 16.7±11.2% (*n*=8), 5.3% (*n*=1) for Cd, Pb and Zn respectively.

II.4. Soil chemical analysis

Environmental availability was estimated by seven chemical methods chosen for their ability to extract the metal in four different soil fractions and used commonly in bioavailability assessment procedures (Pueyo et al., 2004; Bur et al., 2010): (i) the total metal concentration using strong acid extraction, (ii) the metal strongly bound to soil particles using an organic extractant, (iii) the metal weakly bound to soil particles using neutral salts and (iv) the metals present in soil solution using predictive multivariate equations.

II.4.a. Strong acid extractant

- Total metal concentration: total Cd, Pb and Zn measurements were made by inductively-coupled plasma atomic emission spectrophotometry (ICP-AES) after digestion of the soil samples (250 mg) with hydrofluoric and perchloric acids as described in (AFNOR, 1996).

II.4.b. Organic extractant

- Ethylene diamine tetraacetic acid (EDTA) extraction: EDTA is used to mobilize and remove metals as water-soluble metal-EDTA complexes (Udovic and Lestan, 2009). This technique is able to remove metals which are strongly bound to the soil fraction, leaving the residual metals in the non-labile pool (primary minerals). EDTA solution (0.05 M, Carlo Erba, analytical quality) adjusted to pH 7 was added to the soil sample (1/10 s:s) and shaken end-over-end at 30 rpm for 1 hour. Then, the extracts were separated from the solid residue by centrifugation at 3000g for 10 minutes.

II.4.c. Neutral salt extractants

The neutral salt extracts (NH_4NO_3, $NaNO_3$ and $CaCl_2$) are extracted by cationic exchangers. They work by exchanging cations with metals on the exchange complex.

- Calcium chloride ($CaCl_2$) extraction: soil extracts were obtained after shaking $CaCl_2$ solution (0.01 M, Carlo Erba, analytical quality) and soil (1/10 s:s) in an end-over-end shaker for 2 h at 30 rpm as described by Pueyo et al. (Pueyo et al., 2004).
- Ammonium nitrate (NH_4NO_3) extraction: soil extracts were obtained after shaking NH_4NO_3 solution (1 M, Carlo Erba, analytical quality) and soil 1/10 s:s) in an end-over-end shaker for 2 h at 60 rpm. Then the extracts were separated from the solid residue by centrifugation at 3000g for 10 minutes. The supernatant was filtered through a 0.45µm membrane as described by Pueyo et al. (Pueyo et al., 2004).
- Sodium nitrate ($NaNO_3$) extraction: soil extracts were obtained after shaking $NaNO_3$ solution (0.1 M, Carlo Erba, analytical quality) and soil (1/2.5 s:s) in end-over-end shaker for 1 h at 60 rpm. Then the extracts were separated from the solid residue by centrifugation at 3000g for 10 minutes. The supernatant was filtered through a 0.45µm-membrane as described by Pueyo et al. (Pueyo et al., 2004).

Then, supernatants were acidified (0.5%HCl, Carlo Erba, analytical quality) to prevent metal adsorption on vessel and stored at 4°C before analysis by atomic absorption spectrometry with a graphite furnace (GFAAS, Varian 220Z with Zeeman background correction).

II.4.d. Estimation of the metal in the soil solution

- Free metal ion activity: this method is generally used to predict metal assimilation by plants (Bell et al., 1991). It was calculated using the equations of Ge et al. (Ge et al., 2000) which estimate the free metal ion activity as a function of total soil concentration and pH.
- Total metal concentration dissolved in soil solution: it was calculated using the equations of Sauvé et al. (Sauvé et al., 2000) which estimate the total dissolved metal concentration in soil solution as a function of total soil concentration, OM content and pH. Total dissolved metals in the soil solution are assumed to be easily bioavailable for organisms (Barber, 1995).

II.5. Statistical analysis and modelling

II.5.a. Accumulation kinetics

Bioavailability results from a dynamic interaction between the metal in soil (environmental availability, EA) and the species physiology (Table 21). For its assessment, a one-compartment model was used to fit the accumulation kinetics data (Peijnenburg et al., 1999a; Gimbert et al., 2006). This model expresses the dynamic change of tissue metal concentration over time (Eq. 1):

$$C_{sn}(d) = C_{sn}(0) + \frac{a}{k_2}\left(1 - e^{-k_2 t}\right) \quad (1)$$

where a = assimilation flux constant (Table 21), which is representative of EB (Gimbert et al., 2006) ($mg_{metal}.kg\ DW_{sn}^{-1}.d^{-1}$); t = time (days); C_{sn} = metal concentration in the snail viscera ($mg.kg_{sn}^{-1}$ DW); k_2 = the excretion rate constant (d^{-1}). $C_{sn}(0)$ is the metal concentration measured in six snails at the beginning of the experiment.

The accumulation and elimination parameters were estimated by fitting the models with a non-linear mixed-effects procedure (nlme) allowing nested random effects. The within-group errors were allowed to be correlated and/or to have unequal variances. The nlme procedure integrates the soil as a fixed factor and the container as a random effect. Significance (i.e. difference from zero) of the modelled kinetic parameters was assessed using p-values (ANOVA, $p<0.05$). Statistical differences in

parameter estimates between treatments were judged from the absence of overlap of their 95% confidence intervals (95% CI).

II.5.b. Relating environmental bioavailability (a) and environmental availability (C_x) to soil parameters

To determine the influence of soil parameters on EB, to check the influence of total soil concentration on EA and to find which soil parameters influence metal extractability (Table 21), multivariate regression of EB (a) against soil parameters and mono/multivariate regressions using chemical estimation of EA (C_x) as a function of (i) total soil concentration and (ii) total soil concentration coupled with soil characteristics were performed (Eq. 2):

(2) $$Y = x * A + y * B +,..., z$$

where, Y = a or C_x or $\log(k_1)$; x ,y,… represent the coefficients and A, B, … represent the total soil metal concentrations or extracted metal concentrations and the pH and the log transformed soil parameter (OM and clay contents, CEC, aluminium and iron oxides) (Pauget et al., 2011). The best model was chosen according to (i) the highest determination coefficient (r^2_{adj}) and (ii) parsimony.

Table 21: Definition of the concepts used and parameters modulating metal bioavailability

Aspect of bioavailability	Parameter measured	Unit	Definition
Environmental availability (EA)	C_x	$mg_{metal}.kg_{soil}^{-1}$	Physico-chemical processes which govern metal partition between solid and liquid phases of soil.
Environmental bioavailability (EB)	assimilation flux (a)	$mg_{metal}.kgDW_{org}^{-1}\ d^{-1}$	Physiological dynamic processes which control assimilation of the pollutant by the organism (i.e. metal crossing the biological membranes).
Exposure	Uptake rate ($k_{1)}$	$kg_{soil}.kgDW_{org}^{-1}\ d^{-1}$ or $L.kgDW_{org}^{-1}.d^{-1}$	k_1 = Ratio a/Cx representing the exposure of the organisms to contaminant in soil.
Modulating parameter studied	soil properties		Total soil concentration (C_{tot}), pH, organic matter (OM) and clay contents, cation exchange capacity (CEC), aluminium (Al_{ox}) and iron (Fe_{ox}) oxides.

Selection of chemical method to assess and predict environmental bioavailability

The efficiency of an extractant to assess and predict EB was checked by deriving an uptake rate constant (k_1), allowing the influence of soil parameters to be compared with both EB (a) and EA (C_x) (Eq. 3):

(3) $$k_{1(x)} = \frac{a}{C_x}$$

Insofar as the assimilation flux (a) is constant over the duration of exposure, the units in which the uptake rate (k_1) is expressed depends on the units of the EA (C_x) estimated according to (1) the total soil concentration (C_{tot} in mg.kg DW_{soil}^{-1}), (2) the EDTA-extractable metal concentration (C_{EDTA} in mg.kg DW_{soil}^{-1}), (3) the $CaCl_2$-extractable metal concentration (C_{CaCl2} in mg.kg DW_{soil}^{-1}), (4) the NH_4NO_3-extractable metal concentration (C_{NH4NO3} in mg.kg DW_{soil}^{-1}), (5) the $NaNO_3$-extractable metal concentration (C_{NaNO3} in mg.kg DW_{soil}^{-1}), (6) the free ion metal activity (C_{ion} in $mol.L^{-1}$) and (7) the total metal dissolved in the soil solution (C_{diss} in $\mu g.L^{-1}$). The uptake rate (k_1) is expressed

in $kg_{soil}.kg\ DW_{sn}^{-1}.d^{-1}$ (for total metal concentration, EDTA, $CaCl_2$, NH_4NO_3 and $NaNO_3$ extraction) or in $L.kg\ DW_{sn}^{-1}.d^{-1}$ (for total dissolved metal in the soil solution and free ion metal activity).

To determine whether a chemical method is suitable to assess EB (Figure 38), the multivariate regression of uptake rates (k_1) against soil characteristics was performed. As shown by Pauget et al. (Pauget et al., 2011), a chemical method is potentially usable when the regression is not significant. Indeed, absence of a significant influence of soil characteristics on uptake means that the influence of soil parameters is nearly similar on the extractable concentration and on EB. This does not mean that EA and EB are necessarily influenced by the same parameters, but it indicates that the extractant tested may be a potential indicator of EB. After this first screening, correlation between EB (a) and chemical estimation of EA (C_x) were checked using monovariate regressions for all extractants. Among the pre-selected methods using the uptake rate, the one which presents the greatest correlation with the EB was chosen. To predict EB, multivariate regressions of assimilation fluxes (a) against the chemical estimation of EA coupled with soil characteristics were performed.
NB. The influence of soil properties on the metal in soil solution (free ion metal activity and total metal concentration dissolved on soil solution) was not performed to prevent circular reasoning because these two estimations of EA were calculated by using the soil properties.

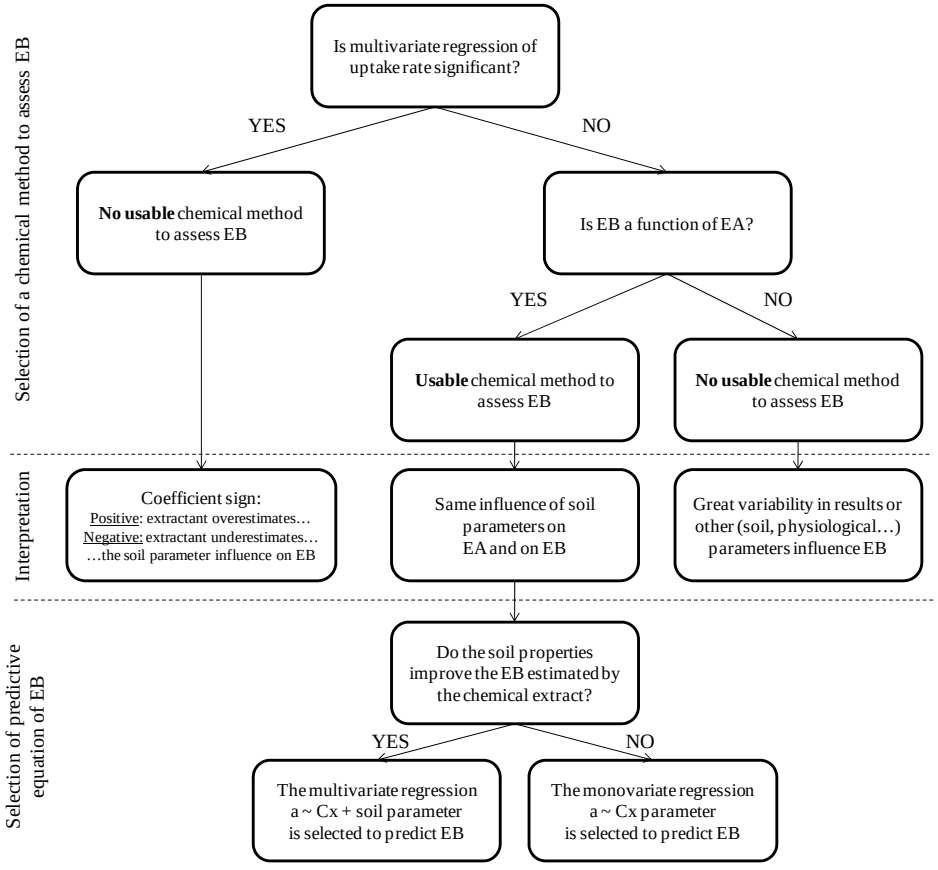

Figure 38: Decision tree to select a chemical method to assess and predict bioavailability. EA: environmental availability, EB: environmental bioavailability

All statistics were performed with the free statistics software package R (version 2.10.1, (R Development Core Team, 2011)).

III. Results

III.1. Chemical method extraction efficiency

The comparison between the extractable metal capacities of the different chemical methods (EDTA, NH_4NO_3, $NaNO_3$ and $CaCl_2$) is shown in Table 22. The EDTA approach exhibited the highest extraction yields for the metals studied (64.8±18.6% of Cd; 55.9±22.7% of Pb and 27.9±11.5% of Zn). These results are in accordance with Udovic et al. (Udovic et al., 2007), who found 68% of Cd, 58% of Pb and 25% of Zn removal from agricultural soils.

Unlike chelating extractants (such as EDTA), neutral salts remove the metal from the soil solid phase by swamping the soil with the desorbing cation (McLaughlin et al., 2000). The neutral salts have lower extraction capacities than EDTA and some differences were observed between the three metals. For Cd and Pb, the $CaCl_2$ and the NH_4NO_3 extracts were the most efficient and showed the same extraction capacities (about 3.5 and 0.1% on average for Cd and Pb, Table 22). For Zn, the NH_4NO_3 extraction ability was the highest (7.30±10.97%) as for Cd and Pb but the $CaCl_2$ is the least efficient extraction method with only 1.71±2.56 % of total Zn concentration.

III.2. Estimation of the assimilation fluxes and excretion rates

After 28 days of exposure, only 2.7% snails have died showing the absence of letality of soil contamination. Accumulation kinetics allow the modelling of two key parameters of transfer, EB (a: assimilation flux) and the excretion rate (k_2). For Cd and Pb, an increase of the metal concentrations in the snail viscera was observed for every soil during the exposure time (Table 23). This is shown by the significant assimilation fluxes which ranged from 0.21 to 1.30 and from 1.38 to 171 $mg_{metal}.kg_{sn}^{-1}d^{-1}$ for Cd and Pb, respectively. However for Zn, only 11 (out of 17) assimilation fluxes were significant, ranging from 9.11 to 273 $mg_{metal}.kg_{sn}^{-1}.d^{-1}$. The absence of significant assimilation flux for Zn could be linked to the poor Zn availability in these soils. Indeed, soils A, E, K, X, Y and Z, on which snails had no significant assimilation fluxes, presented some of the lowest total and extracted Zn concentrations (Table 22).

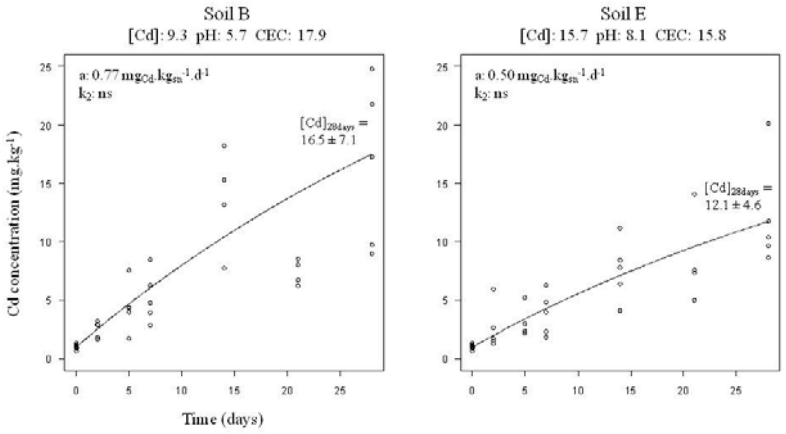

Figure 39 : Accumulation kinetics of Cd in *C. aspersus* snails exposed to soils G and K. Each data point represents an individual snail. ns: not significant; a: assimilation flux ($mg_{metal}.kg_{sn}^{-1}.d^{-1}$); k_2: excretion rate (d^{-1}), $[Cd]_{28days}$: Cd concentration in snails viscera after 28 days of exposure ($mg_{metal}.kg_{sn}^{-1}$).

The accumulation patterns of these three metals were different. For Cd, a quite linear pattern (Figure 39) was observed as previously shown by Gimbert et al. (Gimbert et al., 2006). This suggests the absence of Cd excretion by snails during the exposure, as reflected by a low modelled value of k_2 (Table 23). Concerning Pb, non-linear accumulation patterns achieving or tending to equilibrium were observed (Figure 40). This stabilization of accumulation seems to result from the significant excretion of Pb as shown by values of k_2 (Table 23). In five soils (E, J, K, Y, Z) the excretions were not significant, probably due to the weakness of the associated assimilation fluxes. For Zn, the two previously described patterns were observed (Figure 41). Almost linear patterns of accumulation were found for the snails exposed to most soils, suggesting an absence of Zn excretion during exposure (Table 23).
However, snails exposed to soils C and N presented accumulation patterns tending to equilibrium (Figure 41) in relation with a significant Zn excretion by snails during the exposure (Table 23). These Zn excretions were coupled with the highest assimilation fluxes (Table 23) suggesting that the Zn in excess of the metabolic requirements has been excreted.

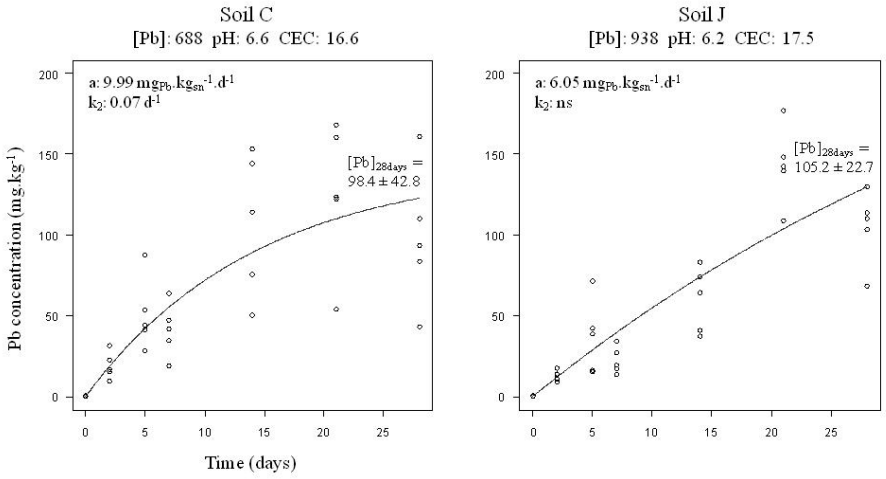

Figure 40: Accumulation kinetics of Pb in *C. aspersus* snails exposed to soils D and E. Each data point represents an individual snail; a: assimilation flux ($mg_{metal}.kg_{sn}^{-1}.d^{-1}$); k_2: excretion rate (d^{-1}), $[Pb]_{28days}$: Pb concentration in snails viscera after 28 days of exposure ($mg_{metal}.kg_{sn}^{-1}$).

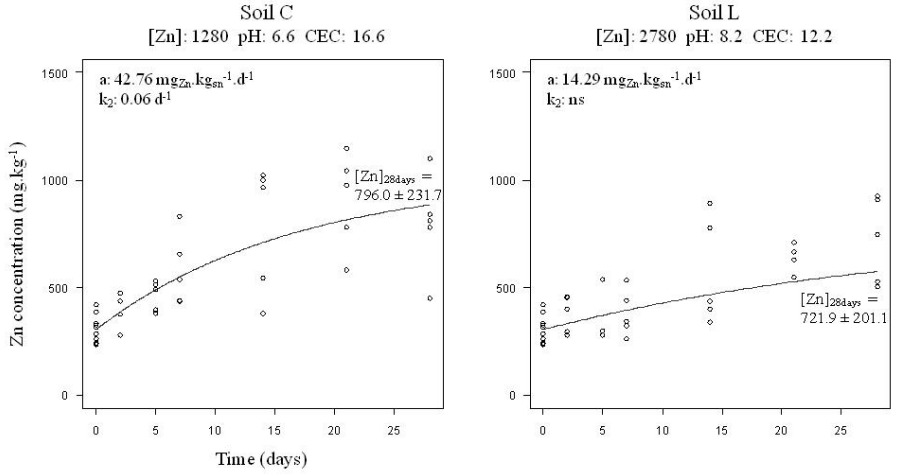

Figure 41: Accumulation kinetics of Zn in C. aspersus snails exposed to soils C and K. Each data point represents an individual snail; a: assimilation flux ($mgmetal.kgsn^{-1}.d^{-1}$); k_2: excretion rate (d^{-1}), $[Zn]_{28days}$: Zn concentration in snail viscera after 28 days of exposure ($mg_{metal}.kg_{sn}^{-1}$).

Table 22: Estimates of environmental availability (Cx, $mg_{metal}.kg_{soil}^{-1}$) using the seven chemical methods for Cd, Pb and Zn. Ion: free ion activity; Diss: total dissolved metal concentration in soil solution. ± SD (n=3).

Soil	Measured concentration					Calculated concentration	
	Total	EDTA	NH_4NO_3	$NaNO_3$	$CaCl_2$	Ion	Diss
	($mg.kg^{-1}$)	($mg.kg^{-1}$)	($mg.kg^{-1}$)	($mg.kg^{-1}$)	($mg.kg^{-1}$)	($mol.L^{-1}$)	($\mu g.L^{-1}$)
Cadmium							
A	6.4	4.17±0.37	0.08±0.00	0.02±0.00	0.08±0.00	6.53E-10	0.19
B	9.3	8.18±0.06	1.11±0.08	0.27±0.02	1.13±0.01	1.99E-09	2.00
C	15.1	13.8±0.38	1.01±0.08	0.25±0.01	1.12±0.08	1.23E-09	1.68
D	23.1	17.1±0.57	0.50±0.03	0.12±0.01	0.79±0.01	8.08E-10	0.61
E	15.7	10.5±0.39	0.21±0.04	0.03±0.00	0.16±0.01	5.15E-10	0.32
F	32.1	21.8±0.49	0.47±0.02	0.09±0.01	0.45±0.01	6.27E-10	0.71
G	38.6	26.2±0.17	1.50±0.05	0.24±0.02	1.65±0.01	6.76E-10	1.35
H	22.4	18.5±0.33	1.68±0.05	0.46±0.02	1.73±0.02	2.28E-09	3.52
I	28.1	21.3±0.43	0.66±0.02	0.15±0.00	0.98±0.06	1.07E-09	0.71
J	13.1	10.4±0.34	0.55±0.01	0.16±0.01	0.84±0.02	1.53E-09	1.38
K	8.1	3.96±0.31	0.05±0.01	0.03±0.03	0.10±0.07	7.58E-10	0.07
L	13.5	3.68±0.20	1.19±0.24	0.01±0.00	0.06±0.01	4.87E-10	0.12
M	11	5.07±0.14	0.09±0.00	0.01±0.00	0.07±0.00	5.13E-10	0.07
N	80.8	20.8±0.47	0.53±0.09	0.03±0.00	0.31±0.04	5.07E-10	0.59
X	17.4	10.6±0.29	0.19±0.01	0.03±0.00	0.19±0.02	5.69E-10	0.40
Y	6.8	4.52±0.28	0.31±0.01	0.05±0.00	0.13±0.00	9.39E-10	0.70
Z	3.1	2.08±0.08	0.03±0.00	0.01±0.00	0.09±0.00	5.62E-10	0.09
Lead							
A	683	162±9	0.14±0.04	0.15±0.00	0.17±0.16	3.16E-11	3.7
B	690	562±12	5.35±0.30	1.89±0.28	2.42±0.38	1.55E-09	19.2
C	688	581±71	5.62±0.20	0.60±0.04	0.43±0.43	2.88E-10	9.40
D	1570	1095±12	1.08±0.06	0.87±0.17	0.47±0.27	1.38E-10	8.00
E	671	208±13	0.14±0.03	0.06±0.01	0.19±0.20	1.37E-11	2.6
F	1680	1326±43	0.61±0.02	0.38±0.05	0.33±0.07	6.07E-11	5.7
G	1920	1324±20	0.85±0.14	0.32±0.04	1.29±1.11	8.86E-11	6.9
H	2120	1392±44	7.98±0.45	4.29±0.31	3.32±0.39	6.62E-09	43.8
I	2450	1333±49	0.85±0.19	0.27±0.03	0.25±0.02	5.45E-10	15.6
J	938	595±25	1.17±0.06	0.83±0.07	0.60±0.10	8.11E-10	15.4
K	524	253±10	0.09±0.02	0.06±0.03	0.84±1.05	4.22E-11	3.9
L	2890	569±8	0.20±0.01	0.10±0.03	0.73±0.11	4.05E-11	5.3
M	2030	519±11	0.26±0.00	0.17±0.06	0.07±0.03	3.56E-11	4.7
N	37100	9461±322	3.88±0.43	0.53±0.06	1.83±0.70	4.41E-10	23.5
X	911	504±3	0.51±0.15	0.31±0.04	0.63±0.44	2.52E-11	3.5
Y	392	309±19	0.44±0.03	0.07±0.02	0.05±0.00	6.85E-11	4.6
Z	159	119±1	0.03±0.03	0.02±0.00	0.05±0.01	5.22E-12	1.6
Zinc							
A	1030	183±9	7.9±1.7	4.6±0.1	0.7±0.3	3.48E-08	62.4
B	677	241±14	244.9±3.9	67.9±3.7	52.4±2.5	9.63E-08	458
C	1280	653±6	274.3±5.8	88.4±2.4	70.9±1.6	8.80E-08	320
D	1560	578±34	65.0±1.8	25.7±1.2	19.9±0.9	6.19E-08	116
E	854	233±6	8.6±4.0	2.2±0.2	1.0±0.4	2.27E-08	30.9
F	2050	653±23	32.8±0.1	9.9±0.7	6.6±0.4	5.63E-08	96.5
G	2830	703±21	81.8±7.1	18.3±1.0	20.6±2.6	7.90E-08	177
H	1080	491±40	300.3±10.0	98.8±5.4	68.8±1.1	1.62E-07	738
I	2850	834±29	115.0±8.9	36.8±1.1	35.6±1.0	1.38E-07	301
J	870	372±14	128.8±4.7	46.9±1.5	37.8±1.8	8.50E-08	294
K	660	181±12	9.2±0.1	3.0±1.0	4.0±2.9	2.96E-08	30.2
L	2780	304±3	17.8±3.5	2.6±0.1	1.6±0.6	5.26E-08	64
M	1930	295±16	13.4±1.0	3.1±0.5	1.5±0.0	4.22E-08	43.4
N	33700	5446±800	67.8±0.5	6.7±1.4	8.1±0.6	3.77E-07	660
X	1092	202±4	8.7±2.5	2.7±0.3	1.5±0.3	3.09E-08	47.5
Y	430	106±4	20.3±0.4	4.8±0.2	1.8±0.7	2.75E-08	75.1
Z	287	54±0	2.9±0.4	0.9±0.5	< LD	1.09E-08	15

Table 23: Kinetic parameter estimates for Cd, Pb and Zn accumulation in C. aspersus snails exposed to the contaminated soils. a: assimilation flux (mgmetal.kg_{sn}^{-1}.d^{-1}); k_1: uptake rate (kg_{soil}.kg_{sn}^{-1}.d^{-1} or L.kg_{sn}^{-1}.d^{-1}); C_x: environmental availability (mg_{metal}.kg_{soil}^{-1}); k_2: excretion rate (d^{-1}); 95%CI: 95% confidence interval.

Soil	$a = k_1 * C_x$ $mg_{metal}.kg_{sn}^{-1}.d^{-1}$	95% CI	p-value	k_2 d^{-1}	95% CI	p-value	r^2
			Cadmium				
A	0.23	0.20/0.27	<0.001	0.04	0.01/0.06	0.182	0.7
B	0.77	0.65/0.88	<0.001	0.02	0.00/0.05	0.414	0.56
C	0.94	0.80/1.09	<0.001	0.03	0.01/0.04	0.204	0.72
D	0.9	0.78/1.02	<0.001	0.03	0.01/0.04	0.237	0.73
E	0.5	0.40/0.60	<0.001	0.02	0.00/0.04	0.397	0.76
F	0.72	0.64/0.80	<0.001	0	0.00/0.01	0.107	0.73
G	1.01	0.76/1.25	<0.001	0	0.00/0.01	0.649	0.61
H	0.67	0.57/0.77	<0.001	0	0.00/0.00	0.139	0.8
I	0.84	0.70/0.97	<0.001	0.04	0.02/0.07	0.146	0.62
J	0.58	0.49/0.66	<0.001	0.01	0.00/0.03	0.682	0.7
K	0.27	0.22/0.31	<0.001	0.04	0.01/0.06	0.148	0.77
L	0.41	0.30/0.52	0.002	0.08	0.04/0.13	0.079	0.7
M	0.46	0.38/0.55	<0.001	0	0.00/0.03	0.933	0.49
N	1.3	1.12/1.48	<0.001	0.03	0.01/0.06	0.147	0.78
X	0.78	0.64/0.91	<0.001	0.05	0.02/0.08	0.13	0.54
Y	0.37	0.32/0.42	<0.001	0.03	0.01/0.05	0.139	0.77
Z	0.21	0.18/0.24	<0.001	0.05	0.02/0.07	0.055	0.76
			Lead				
A	7.56	6.57/8.55	<0.001	0.06	0.03/0.08	0.041	0.85
B	10.9	8.27/13.5	<0.001	0.07	0.04/0.09	0.04	0.93
C	9.99	8.48/11.5	<0.001	0.07	0.05/0.09	0.004	0.78
D	22.03	17.7/26.4	<0.001	0.12	0.08/0.16	0.013	0.88
E	5.38	4.22/6.53	<0.001	0.02	0.00/0.04	0.51	0.8
F	19.76	16.9/22.6	<0.001	0.05	0.03/0.07	0.039	0.72
G	21.39	17.2/25.6	<0.001	0.07	0.05/0.10	0.014	0.82
H	19.2	15.9/22.5	<0.001	0.07	0.04/0.10	0.042	0.67
I	18.48	14.3/22.7	<0.001	0.12	0.07/0.17	0.029	0.61
J	6.05	5.19/6.90	<0.001	0.02	0.00/0.04	0.288	0.79
K	5.83	4.37/7.30	<0.001	0.05	0.01/0.09	0.051	0.37
L	37.22	29.2/45.2	<0.001	0.17	0.11/0.22	0.009	0.57
M	14.94	12.2/17.8	<0.001	0.06	0.03/0.09	0.047	0.65
N	171.24	141/201	<0.001	0.15	0.11/0.19	0.047	0.64
X	17.3	14.4/20.2	<0.001	0.1	0.07/0.13	0.008	0.74
Y	3.26	2.71/3.81	<0.001	0.01	0.00/0.04	0.571	0.69
Z	1.38	1.19/1.57	<0.001	0	0.00/0.01	0.549	0.8
			Zinc				
A	12.22	5.23/19.2	0.116	0.04	0.00/0.11	0.592	0.26
B	21.78	13.6/30.0	0.021	0.08	0.03/0.13	0.169	0.73
C	42.76	33.5/52.0	<0.001	0.06	0.03/0.08	0.035	0.7
D	23.03	17.1/29.0	0.002	0.04	0.01/0.06	0.254	0.82
E	9.8	4.56/15.0	0.096	0.01	0.00/0.06	0.837	0.72
F	19.01	13.0/25.0	0.008	0	0.00/0.03	0.946	0.74
G	24.8	18.0/31.6	0.003	0.01	0.00/0.04	0.693	0.81
H	27.56	20.5/34.6	0.002	0.03	0.00/0.06	0.388	0.77
I	9.11	5.40/12.8	0.033	0	0.00/0.00	0.208	0.79
J	9.83	6.30/12.4	0.016	0	0.00/0.01	0.516	0.78
K	4.54	0.43/8.7	0.313	0	0.00/0.05	0.765	0.21
L	14.29	9.34/19.2	0.013	0	0.00/0.03	0.913	0.66
M	11.6	6.64/16.6	0.04	0	0.00/0.02	0.639	0.64
N	273.27	228/318	<0.001	0.07	0.04/0.09	0.03	0.81
X	3.1	1.16/5.03	0.14	0	0.00/0.00	0.173	0.49
Y	0.1	0.00/0.09	0.899	0	0.00/0.00	0.36	0.24
Z	0.33	0.00/1.74	0.831	0	0.00/0.12	0.662	0.05

III.3. Determination of chemical methods to assess and predict environmental bioavailability

During statistical analysis, we observed that soil N, which was highly contaminated (Table 20) and on which snails presented high assimilation fluxes (Table 23), tended to bias the regressions. Therefore, soil N was removed from the data for this part of the interpretation of results.

III.3.a. Influence of soil properties on environmental bioavailability

By relating assimilation fluxes to total soil concentration and soil parameters (Table 24), we identified total concentrations, pH, CEC and oxides of aluminium and iron as the main parameters modulating EB of Cd, Pb and Zn to snails (Table 24). Indeed, the elevated determination coefficients of the regressions (0.81, 0.83 and 0.75 for Cd, Pb and Zn, respectively) coupled with significant descriptors attest to the soil parameters influence on metal EB to snails.

III.3.b. Influence of soil properties on environmental availability

The EDTA-extracted metal concentrations were correlated to the total soil concentrations for the three metals. For Cd, an elevated determination coefficient (0.91) was observed compared to Pb and Zn (0.45 and 0.48, respectively) (Table 25). Cadmium was the only metal for which total soil concentration explained a significant part of neutral-salts-extracted concentration even if low determination coefficients were observed (0.21 and 0.32 for NH_4NO_3 and $CaCl_2$, respectively). The addition of soil parameters gave a better explanation of the EA estimated by the chemical extracts as shown by the higher determination coefficients of the multivariate regressions (Table 25). Soil pH was identified as the most important parameter influencing EA. Indeed in 92% of cases, a significant influence of pH was observed (Table 25). To a lesser extent, CEC and iron oxide also influenced the EA. Iron oxide was identified as influencing concentrations in metal extracted by EDTA.

III.3.c. Selection of a chemical method to assess metal environmental bioavailability to snails

From screening the uptake rate regressions (Table 26), two chemical methods seemed suitable to assess Cd and Pb EB to snails: the total metal concentration in soil and the EDTA extracted concentration. The uptake rate variation based on these two methods could not be explained by the soil parameters suggesting a similar influence of soil parameters on EA and on EB. The uptake rates based on the other chemical methods were significantly explained by the soil characteristics with *p-value* (<0.007) showing a different influence of soil characteristics on EA and on EB. Thus, the NH_4NO_3, $NaNO_3$, $CaCl_2$, free ion activity and total dissolved concentration are not suitable methods to assess and predict Cd and Pb EB. To discriminate between the two chemical methods selected, we focussed on their ability to explain EB (*i.e.* assimilation flux). For Cd, the EDTA extract is more suitable than the total soil concentration as testified by its higher determination coefficient (r^2_{adj} = 0.67 against 0.56), while for Pb, the total soil concentration seems to be the best chemical method (r^2_{adj} = 0.77 against 0.32). For Zn, the screening showed that none of the chemical methods were usable (all regressions were significant). This was confirmed when relating assimilation fluxes and extracted concentrations using the different chemical methods, where no regressions were significant (Table 26).

Table 24: Influence of soil characteristics on Cd, Pb and Zn environmental bioavailability (a, assimilation flux) to snails estimated using multivariate regressions. Statistical significance: °: p-value<0.1; *: p-value<0.05; **: p-value<0.01; ***: p-value<0.001.

Metal	Equation	p-value	r^2_{adj}
Cd	a = 2.86±0.69** + 0.02±0.00 Cd_{tot}*** - 0.14±0.04 pH** - 1.24±0.39 log CEC** - 0.26±0.13 log Fe_{ox}°	<0.001	0.81
Pb	a = 35.8±14.0* + 0.01±0.00 Pb_{tot}*** - 27.5±11.0 log CEC*	<0.001	0.83
Zn	a = - 37.3±20.3 + 0.03±0.01 Zn_{tot}** - 23.0±5.2 pH** - 183±37.4 log Al_{ox}** - 13.79±8.15 log Fe_{ox}	0.023	0.75

Table 25: Influence of (i) total soil concentration and (ii) total soil concentration coupled with soil parameters on environmental availability (C_x) estimated by the chemical extractants (EDTA, NH_4NO_3, $NaNO_3$ and $CaCl_2$) using multivariate regressions for Cd, Pb and Zn. Statistical significance: °: p-value<0.1; *: p-value<0.05; **: p-value<0.01; ***: p-value<0.001. ns: no significant regression.

Metal	Method	Equation	p-value	r^2_{adj}
Cd	EDTA	Cd_{EDTA} = -0.66±1.11 + 0.73±0.06 Cd_{tot}	<0.001	0.91
		Cd_{EDTA} = 10.8±2.69** + 0.73±0.03 Cd_{tot}*** - 1.61±0.36 pH*** - 4.06±1.24 log Fe_{ox}**	<0.001	0.98
	NH_4NO_3	Cd_{NH4NO3} = 0.14±0.23 +0.03±0.01 Cd_{tot}*	0.04	0.21
		Cd_{NH4NO3} = 5.26±0.91*** + 0.03±0.01 Cd_{tot}** - 0.49±0.08 pH*** - 0.79±0.27 log OM* + 1.48±0.34 log Fe_{ox}**	<0.001	0.78
	$NaNO_3$	Cd_{NaNO3} = ns	ns	ns
		Cd_{NaNO3} = 0.94±0.12*** + 0.005±0.001 Cd_{tot}** - 0.12±0.02 pH***	<0.001	0.83
	$CaCl_2$	Cd_{CaCl2} = 0.01±0.24+ 0.04±0.01 Cd_{tot}*	0.013	0.32
		Cd_{CaCl2} = 3.49±0.54*** + 0.03±0.01 Cd_{tot}*** - 0.48±0.07 pH***	<0.001	0.83
Pb	EDTA	Pb_{EDTA} = 185±160+ 0.39±0.11 Pb_{tot}**	0.003	0.45
		Pb_{EDTA} = 182±169 + 0.67±0.12 Pb_{tot}*** - 1290±403 log Fe_{ox}**	<0.001	0.67
	NH_4NO_3	Pb_{NH4NO3} = ns	ns	ns
		Pb_{NH4NO3} = 18.3±3.24*** + 0.0003±0.000 Pb_{tot} - 2.36±0.44 pH***	<0.001	0.64
	$NaNO_3$	Pb_{NaNO3} = ns	ns	ns
		Pb_{NaNO3} = 7.44±1.49*** + 0.0003±0.0002 Pb_{tot} - 1.00±0.20 pH***	<0.001	0.61
	$CaCl_2$	Pb_{CaCl2} = ns	ns	ns
		Pb_{CaCl2} = 5.88±1.45** + 0.0002±0.0002 Pb_{tot} - 0.76±0.20 pH**	0.005	0.49
Zn	EDTA	Zn_{EDTA} = 101±84.2 + 0.20±0.05 Zn_{tot}**	0.02	0.48
		Zn_{EDTA} = 735±269* + 0.30±0.04 Zn_{tot}*** - 107±36.7 pH* - 407±155 log Fe_{ox}*	<0.001	0.77
	NH_4NO_3	Zn_{NH4NO3} = ns	ns	ns
		Zn_{NH4NO3} = 860±104*** + 0.02±0.01 Zn_{tot} - 112±14.8 pH***	<0.001	0.79
	$NaNO_3$	Zn_{NaNO3} = ns	ns	ns
		Zn_{NaNO3} = 275±33.3*** + 0.006±0.004 Zn_{tot} – 35.7±4.72 pH***	<0.001	0.79
	$CaCl_2$	Zn_{CaCl2} = ns	ns	ns
		Zn_{CaCl2} = 210±25.3*** + 0.007±0.003 Zn_{tot}° - 27.6±3.59 pH***	<0.001	0.79

Table 26: Multivariate regression formulae relating (i) the uptake rate (k_1) of Cd, Pb and Zn to soil characteristics and (ii) the assimilation flux (a) to environmental availability (C_x) estimated with total soil concentration and chemical extracts. Statistical significance: °: p-value<0.1; *: p-value<0.05; **: p-value<0.01; ***: p-value<0.001. ns: no significant regression. Ion: free ion activity; Diss: total dissolved metal concentration in soil solution.

Metal	Method	Equation	p-value	r^2_{adj}
Cd	Total	$\log k_1$ = ns	ns	ns
		a = 0.27±0.09*** + 0.02±0.00 Cd_{tot}***	<0.001	0.56
	EDTA	$\log k_1$ = ns	ns	ns
		a = 0.28±0.07** + 0.03±0.00 Cd_{EDTA}***	<0.001	0.67
	NH_4NO_3	$\log k_1$ = -4.08±1.35* + 0.49±0.08 pH*** - 1.28±0.54 log Clay* + 2.96±0.83 log CEC** - 1.02±0.27 log Fe_{ox}**	0.001	0.71
		a = 0.43±0.08*** + 0.28±0.01 Cd_{NH4NO3}*	0.017	0.29
	$NaNO_3$	$\log k_1$ = -2.24±0.45*** + 0.44±0.06 pH***	<0.001	0.77
		a = 0.46±0.07*** + 1.21±0.43 Cd_{NaNO3}*	0.01	0.31
	$CaCl_2$	$\log k_1$ = -2.42±0.51*** + 0.37±0.07 pH***	<0.001	0.64
		a = 0.40±0.06*** + 0.33±0.08 Cd_{CaCl2}***	<0.001	0.53
	Ion	$\log k_1$ = 11.1±0.64*** - 1.82±0.52 log CEC**	0.004	0.43
		a = ns	ns	ns
	Diss	$\log k_1$ = -4.01±0.46*** + 0.41±0.05 pH*** + 0.56±0.14 log OM**	<0.001	0.84
		a = ns	ns	ns
Pb	Total	$\log k_1$ = ns	ns	ns
		a = 1.10±2.10 + 0.01±0.00 Pb_{tot}***	<0.001	0.77
	EDTA	$\log k_1$ = ns	ns	ns
		a = 5.53±3.50 + 0.01±0.00 Pb_{EDTA}*	0.014	0.32
	NH_4NO_3	$\log k_1$ = -2.70±0.47*** + 0.55±0.06 pH*** + 0.81±0.22 log Fe_{ox}**	<0.001	0.88
		a = ns	ns	ns
	$NaNO_3$	$\log k_1$ = -1.79±0.40*** + 0.47±0.06 pH*** + 0.67±0.19 log Fe_{ox}**	<0.001	0.88
		a = ns	ns	ns
	$CaCl_2$	$\log k_1$ = -1.02±0.79 + 0.34±0.11 pH**	0.007	0.37
		a = ns	ns	ns
	Ion	$\log k_1$ = 5.98±0.77*** + 0.83±0.05 pH*** - 0.81±0.44 log CEC°	<0.001	0.97
		a = ns	ns	ns
	Diss	$\log k_1$ = -0.83±1.05 + 0.30±0.06 pH*** + 0.26±0.16 log OM - 1.35±0.60 log CEC*	<0.001	0.77
		a = ns	ns	ns
Zn	Total	$\log k_1$ = 3.25±6.42 + 0.88±0.28 pH* - 4.53±1.33 log OM** - 8.45±3.44 log Clay* + 14.2±4.25 log CEC**	0.018	0.5
		a = ns	ns	ns
	EDTA	$\log k_1$ = 1.25±4.97 + 0.67±0.22 pH* - 2.97±1.03 log OM* - 5.39±2.66 log Clay° + 9.13±3.29 log CEC*	0.035	0.43
		a = ns	ns	ns
	NH_4NO_3	$\log k_1$ = -7.20±1.51*** + 0.55±0.08 pH*** + 1.52±0.74 log CEC° - 1.06±0.53 log Al_{ox}°	<0.001	0.78
		a = ns	ns	ns
	$NaNO_3$	$\log k_1$ = -5.67±1.04*** + 0.33±0.05 pH*** + 0.76±0.35 log Clay° - 1.57±0.39 log Al_{ox}** + 0.71±0.19 log Fe_{ox}**	<0.001	0.87
		a = ns	ns	ns
	$CaCl_2$	$\log k_1$ = -6.58±1.38*** + 0.31±0.06 pH*** + 1.06±0.47 log Clay* - 2.13±0.51 log Al_{ox}** + 0.99±0.25 log Fe_{ox}**	<0.001	0.81
		a = ns	ns	ns
	Ion	$\log k_1$ = 4.58±26.57 - 4.39±1.78 pH** + 15.9±5.50 log OM* + 31.0±14.2 log Clay° - 56.9±17.6 log CEC**	0.018	0.5
		a = ns	ns	ns
	Diss	$\log k_1$ = -0.39±2.69 + 0.74±0.12 pH*** - 1.99±0.56 log OM** -3.92±1.44 log Clay* + 5.96±5.78 log CEC**	<0.001	0.74
		a = ns	ns	ns

III.3.d. Environmental bioavailability prediction

Soil characteristics were added to the selected chemical extracts to predict metal EB to snails. The pH and CEC increased the EDTA prediction of Cd EB as shown by the increase of the determination coefficient rising from 0.67 (Table 26) to 0.82 (Table 27). This showed that, even though the pH already influenced the EDTA extract, its influence on EB was a little different. Moreover, the CEC influence was not taken into account by the EDTA extract (Table 25) while it influenced Cd EB to snails (Table 24). Thus, the equation based on EDTA extraction, pH and CEC (Table 27) was chosen to predict Cd EB. For Pb, the best EB prediction using total soil concentration was obtained when taking into account the CEC influence with a determination coefficient rising from 0.77 (Table 26) to 0.83 (Table 27). Predicted assimilation fluxes of Cd and Pb were in accordance with the measured assimilation fluxes (Figure 42). Significant regressions (p-value < 0.001) were obtained when correlating predicted and measured assimilation fluxes of Cd and Pb with elevated determination coefficients (r^2 = 0.97 and 0.95, respectively, Figure 42).

Even though none of the chemical methods tested could alone assess Zn EB to snails, the addition of soil characteristics allowed the prediction of the assimilation flux variation. The determination coefficients observed ranged from 0.75 (for total metals in soil) to 0.93 (for free ion activity in soil solution) (Table 27). Then, when using the equation with the total soil concentration and the soil characteristics to predict Zn EB (Table 27), the correlation between the measured and calculated assimilation fluxes was significant and presented a high determination coefficient (r^2_{adj} = 0.97, Figure 42). This equation can be used to predict Zn EB to snails.

Table 27: Multivariate regression formulae relating the assimilation flux (a) of Cd, Pb and Zn to environmental availability (C_x) estimated with the chemical extract and soil characteristics. Statistical significance: °: p-value<0.1; *: p-value<0.05; **: p-value<0.01; ***: p-value<0.001. ns: no significant regression. Ion: free ion activity; Diss: total dissolved metal concentration in soil solution.

Metal	Method	Equation	p-value	r^2_{adj}
Cd	Total	a = 2.86±0.69** + 0.02±0.00 Cd_{tot}*** - 0.14±0.04 pH** - 1.24±0.39 log CEC** - 0.26±0.13 log	<0.001	0.81
	EDTA	a = 2.76±0.67** + 0.02±0.00 Cd_{EDTA}*** - 0.12±0.04 pH* - 1.28±0.37 log CEC**	<0.001	0.82
	NH_4NO_3	a = 1.37±0.60* + 0.34±0.08 Cd_{NH4NO3}** + 0.38±0.17 log OM* - 1.39±0.50 log CEC* - 0.78±0.22	0.005	0.62
	$NaNO_3$	a = 2.47±0.63** + 1.49±0.36 Cd_{NaNO3}** - 1.66±0.52 log CEC** - 0.23±0.19 log Fe_{ox}	0.004	0.57
	$CaCl_2$	a = 1.69±0.54** + 0.35±0.07 Cd_{CaCl2}*** - 1.05±0.44 log CEC*	<0.001	0.65
	Ion	a = ns	ns	ns
	Diss	a = 2.35±0.71** + 0.19±0.06 Cd_{diss}** + 0.31±0.19 log OM -2.05±.61 log CEC** - 0.44±0.24 log Fe_{ox}	0.017	0.5
Pb	Total	a = 35.8±14.0* + 0.01±0.00 Pb_{tot}*** - 27.5±11.0 log CEC*	<0.001	0.83
	EDTA	a = 47.36±16.25* + 0.01±0.00 Pb_{EDTA}** - 32.92±13.20 log CEC* + 19.30±4.93 log Fe_{ox}**	<0.001	0.77
	NH_4NO_3	a = 64.50±22.56* + 1.47±0.69 Pb_{NH4NO3}° - 42.94±18.58 log CEC* + 25.26±6.41 log Fe_{ox}**	0.003	0.6
	$NaNO_3$	a = 74.28±22.53** + 3.88±1.52 Pb_{NaNO3}* - 51.04±18.62 log CEC* + 22.67±6.01 log Fe_{ox}**	0.001	0.64
	$CaCl_2$	a = 59.46±22.60* + 3.45±1.80 Pb_{CaCl2}° - 39.05±18.60 log CEC° + 22.05±6.55 log Fe_{ox}**	0.004	0.58
	Ion	a = 82.08±26.00** + 2.52 10^9±1.15 10^9 Pb_{ion}* - 56.67±21.43 log CEC* + 20.56±6.42 log Fe_{ox}**	0.002	0.61
	Diss	a = 78.89±23.47** + 0.42±0.16 Pb_{diss}* - 56.00±19.70 log CEC* + 20.71±6.11 log Fe_{ox}**	0.001	0.64
Zn	Total	a = - 37.3±20.3 + 0.03±0.01 Zn_{tot}** - 23.0±5.2 pH** - 183±37.4 log Al_{ox}** - 13.79±8.15 log Fe_{ox}	0.023	0.75
	EDTA	a = ns	ns	ns
	NH_4NO_3	a = 15.99±43.44 + 0.17±0.03 Zn_{NH4NO3}* + 18.58±3.76 pH** - 34.34±10.42 log OM* -	0.01	0.89
	$NaNO_3$	a = -56.40±27.15° + 0.49±0.08 Zn_{NaNO3}*** + 13.78±2.87 pH** - 19.06±5.39 log OM*	<0.001	0.88
	$CaCl_2$	a = -44.32±38.79 + 0.58±0.15 Zn_{CaCl2}** + 11.79±3.93 pH* -17.66±7.83 log OM°	0.008	0.77
	Ion	a = -134±29.7** + 4.85 10^8±6.54 10^7 Zn_{ion}*** + 11.0±2.11 pH** - 33.3±4.89 log OM** -	0.001	0.93
	Diss	a = ns	ns	ns

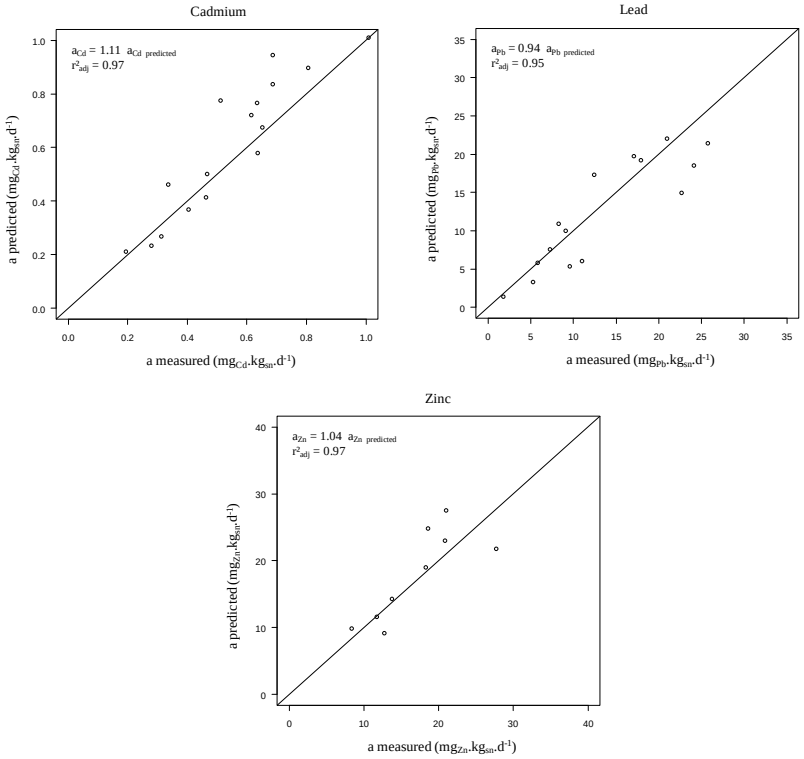

Figure 42: Comparison of calculated and measured assimilation fluxes (a, $mg_{metal}.kg_{sn}^{-1}.d^{-1}$) of Cd, Pb and Zn of snails after 28 days of exposure using the predicted equations of environmental bioavailability. EDTA for Cd, total soil concentration for Pb and Zn, in each equation the soil characteristics are taken into account.

IV. Discussion

IV.1. Determination of a chemical method to assess and predict environmental bioavailability

The influence of soil properties on EA explains the large variations of the extraction capacities and metal concentrations in soil solution we observed between the soils. From a physico-chemical point of view, soil characteristics modulate sorption and mobility of metals in soil (Sterckeman et al., 2004; Zhang et al., 2010; Fonseca et al., 2011; Zeng et al., 2011). For example, it has been shown that an increase of pH leads to a decrease of metal mobility (Sterckeman et al., 2004; Van Gestel and Koolhaas, 2004) and Janssen et al. (Janssen et al., 1997a) pointed out the importance of CEC which is a measure of the amount of available sorption sites. These parameters can influence the transport and the partition of the metals in soil and can thus modify both EA and EB (Janssen et al., 1997b). The multivariate expression relating metal EB to snails and EA to total metal soil concentration and soil parameters showed a significant influence of pH, CEC and iron oxides. This is in accordance with previous observations with other organisms differently exposed to soil contamination, such as collembolans (*Folsomia candida* (Crommentuijn et al., 1997; Van Gestel and Koolhaas, 2004)) and two earthworms (*Eisenia Andrei* and *Lumbricus rubellus* (Peijnenburg et al., 1999c; Veltman et al., 2007)). However, even if the same parameters influence metal EB to several organisms, the intensity of this influence may be variable. Indeed, differences in sources and routes of contamination and physiology between soil organisms play an important role in metal accumulation as underlined by

Schipper et al. (Schipper et al., 2008). For example, snails are mainly exposed by the digestive route (Coeurdassier et al., 2002) whereas the dermal route predominates in the earthworm (Vijver et al., 2003). These differences underline the necessity to assess metal bioavailability for many organisms differently exposed to the soil contaminant.

After the identification of the soil parameters which modulate the EA and EB to snails, these influences were compared using the uptake rates. The correlation between assimilation fluxes and EA allowed the selection of the best chemical method to assess and predict metal EB. This first screening revealed that only total soil concentration and EDTA extract are usable to assess Cd and Pb EB to snail. EDTA extract being correlated with total soil concentration, we assume that Cd and Pb EB to snails is firstly influenced by total soil concentration and secondly by soil parameters. The fact that EDTA extract is correlated with EB confirms that snails are able to pick up the metal in the strongly bound metal phase (Scheifler et al., 2003b) probably in relation with their major route of absorption which is the digestive one (Gomot et al., 1989; Coeurdassier et al., 2002; Gomot-de Vaufleury and Pihan, 2002).

For Cd, EDTA extract was highly correlated to assimilation flux (r^2_{adj} = 0.67) whereas total soil concentration was selected for Pb (r^2_{adj} = 0.77). Even if it has been shown that the $CaCl_2$ extract and metal available in pore water can be used to determine EB to the earthworm (Peijnenburg et al., 1999b; Vijver et al., 2003), several studies have suggested that metal EB was best explained by total metal concentration in soil (Hobbelen et al., 2006; Vijver et al., 2007). In contrast to the work of Houba et al. (Houba et al., 2000), who assumed that $CaCl_2$ could be used as a universal extractant for risk assessment practices, our study showed that EDTA in soil is more suitable than neutral salts for assessment of Cd EB to snails. When soil characteristics were included in the regressions, better fits were obtained whatever the metal considered. Indeed, adding soil pH and CEC to obtain a better forecast of the Cd EB increased the determination coefficient from 0.67 to 0.82, allowing the discrimination of the soil characteristics influencing the EB which are not or not correctly taken into account by the EDTA extract. The CEC influence on EB that is not taken into account by the EDTA extract is identified and the pH influence seems to be underestimated. This is in accordance with Zou et al. (Zou et al., 2009) who have shown that EDTA extract is greatly influenced by pH, which modifies the capacity of a chelating agent to extract the metal in soil. Moreover, iron oxides influence the Cd removal due to the poor efficiency of EDTA to extract metals bound to the Fe oxides (Sun et al., 2001; Bermond et al., 2005).

For Pb, the total metal concentration in soil is a better predictor of EB than the EDTA extractable concentration even though EDTA was selected during the first screening (using uptake rate). This suggest that snails are also able to assimilate strongly bound Pb, as demonstrated by Scheifler et al. (Scheifler et al., 2003b) for Cd, explaining the elevated correlation between EB and total Pb concentration in the soil. We can assume that EDTA extraction is a better method to assess Pb EB to snails than neutral salt extraction due to its ability to partially dissolve mineral compounds such as pyromorphite or mimetite potentially present in soils (Bajda, 2011). However, the absence of total solubilisation can explain the poor correlation between assimilation flux and EDTA extract (r^2_{adj} = 0.32). However, as CEC has been identified as a modulating parameter of Pb EB to snails, the amount of soil binding sites has to be taken into account for predictive purposes.

For Zn, no chemical method was selected to assess EB to snails. This inability of chemical methods was confirmed by the absence of significant correlation between assimilation fluxes and EA estimated by the chemical extracts. This lack of correlation might be due to the fact that this essential metal can be regulated by invertebrates (Dallinger, 1993; Gimbert and de Vaufleury, 2009).

The chemical methods alone are not able to simulate metal regulation. However, soil parameters act as compensating factors that allow the establishment of predictive equations using multiple chemical methods (total soil concentration, neutral salts and ion in soil solution).
Overall, the present data demonstrate that even under experimental laboratory conditions which do not take into account environmental parameters (*e.g.* rainfall, sunshine, plants as contamination source), no single chemical method can be selected for the assessment of EB of metals in soils to snails. However, in this laboratory experiment the strong correlations between Cd assimilated and EDTA-extract and Pb assimilated and Pb total soil concentration allow the assessment of the bioavailable pool of Cd as corresponding to the Cd extracted by EDTA (26 to 91% of the Cd-total soil concentration) modulated by the soil properties and the bioavailable pool of Pb as the total Pb in soil modulated by the CEC, both of them corresponding to strongly bound metal in the soil.

IV.2. Implications in risk assessment

In our experiment, elevated assimilation fluxes were not necessarily observed in soils presenting the highest metal contaminations. Indeed, the soil parameters influence the metal mobility (Sterckeman et al., 2004) and the organism physiology (pH can have a protective effect on free metal ion toxicity) (Lofts et al., 2004; Bur et al., 2010). Using a classical risk assessment procedure ignoring the influence of soil parameters, with similar soil use, the most contaminated soil would be considered as presenting the highest risk, although its metal EB could be low due to soil parameters. That is why numerous studies (Peijnenburg et al., 2007; Dermont et al., 2008; van Gestel, 2008) deal with the determination of chemical methods taking into account the soil characteristics to assess and predict bioavailability in risk assessment procedures. In our study we have shown that EDTA (coupled with pH and CEC) and to a lesser extent total soil concentration (coupled with CEC, pH and iron oxide) allow the correct prediction of the Cd EB to snails. For Pb only the total soil concentration (coupled to CEC) is usable. For Zn, no unique chemical method was selected but several can be used to predict Zn EB to snails when adding soil characteristics. This difference between metals underlines the difficulty to find a global chemical estimator of EB. Indeed, each metal exhibits different behaviours and a competition between metals for binding sites exists in soil. Moreover, the contrasting fates of metals, as shown in this study and in Gimbert et al. (Gimbert et al., 2008c), make it difficult to determine a single chemical method to assess and predict bioavailability. Another aspect to be taken into account is the side reactions occurring between the chemical method and the soil (ISO 17402, 2008). Indeed, Meers et al. (Meers et al., 2007b) have shown that the NH_4NO_3 extract can overestimate the exchangeable fraction by formation of amine complexes.
As recommended in ISO 17402 (ISO 17402, 2008), much care should be given when relating chemical and biological methods (and even more when the objective is to replace a biological method by a chemical method) for bioavailability assessment. Indeed, as shown in Figure 43, the extracted concentrations and assimilation fluxes are highly correlated within a range of total soil concentrations. The Cd-EDTA extracts are correlated to total soil concentrations and present a linear pattern whereas the assimilation fluxes, even though they are closely correlated to total soil concentrations, present a non-linear pattern. For total Cd concentration in soil up to 26 $mg.kg^{-1}$, a correlation can be found between EDTA extract and EB but, after the plateau appears, the correlation vanishes. Thus, the establishment of chemical methods to predict metal EB has to be used carefully and the workable range of total soil concentrations has to be determined.

Instead of looking for a universal chemical estimator of EB, it may be more interesting to focus on total soil concentration coupled with the influence of soil characteristics to assess and predict EB. During this study, even though no chemical method to assess Zn EB was found, a correct predictor of Zn assimilation was obtained by taking into account soil parameters and total soil concentration (r^2_{adj}= 0.75). The same observations were made with the other metals where 81% and 83% of the variations of the assimilation fluxes of Cd and Pb could have been explained by total soil concentrations coupled with soil characteristics. As recommended by Peijnenburg et al. (Peijnenburg and Jager, 2003a) and van Gestel (van Gestel, 2008), the relevance of the chemical methods allowing the EB to be assessed and predicted has to be improved by taking into account species, metal and soil parameters. There is as yet no chemical method that adequately describes metal EB to snails and it is highly unlikely to find a single estimator of EB relevant to every organism. Yet, information about EB contributes substantially to the improvement of risk assessment procedures. That is why integrated approaches combining both biological and chemical measures have to be performed.

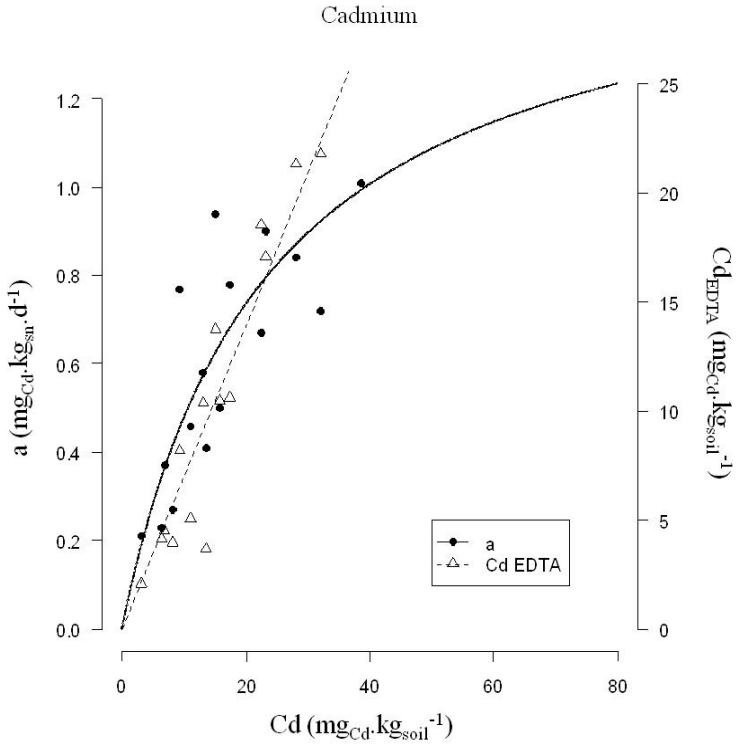

Figure 43: Extract of Cd by EDTA and Cd assimilation fluxes (a, $mg_{metal}.kg_{sn}^{-1}.d^{-1}$) as a function of total soil concentration.

Acknowledgments

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Partie D : Discussion générale, conclusions et perspectives.

I. Discussion générale

Lors de l'ERE liées à la contamination des sol par les métaux, la méthode la plus communément utilisée aujourd'hui en France repose essentiellement sur des mesures de teneurs totales en métaux dans les sols couplées à des durées d'expositions théoriques et des valeurs toxicologiques de référence afin de vérifier si le site est conforme à l'usage que l'on veut en faire (IEM, 2007). Cependant, les transferts de métaux du sol aux organismes sont conditionnés par un grand nombre de paramètres tant physico-chimiques (Peijnenburg et al., 1999c; Calhôa et al., 2011; Pauget et al., 2011) que biologiques (Vijver et al., 2003; Gimbert et al., 2008c). Pour une analyse judicieuse du risque de transfert des métaux, il est donc important de prendre en compte leur biodisponibilité (Fairbrother et al., 2007; van Gestel, 2008; Smith, 2009). Une étude préliminaire à ce travail de thèse, réalisée en conditions contrôlées sur des sols artificiellement contaminés, avait permis de mettre en évidence l'influence du pH et de la MO sur la biodisponibilité du Cd et du Pb chez l'escargot *C. aspersus* (Pauget et al., 2011). Ce travail avait également posé les bases de l'utilisation des taux d'absorption (k_1) pour comparer l'influence des paramètres du sol sur la disponibilité environnementale et la biodisponibilité dans une nouvelle méthodologie. Cette étude avait montré l'incapacité de la concentration totale des métaux dans les sols à prédire la biodisponibilité des métaux. Cependant, ces résultats obtenus en laboratoire devaient être transposés *in situ*.

C'est dans ce cadre d'évaluation de la biodisponibilité des métaux *in situ* et des mécanismes qui la module que s'inscrit ce travail de thèse. L'exposition des escargots sur les 12 sites français du programme Bioindicateur 2 (http://ecobiosoil.univ-rennes1.fr/ADEME-Bioindicateur/) a permis de mettre en évidence l'influence des paramètres des sols et des sources de contamination sur l'accumulation des métaux. Cette exposition a permis également de mettre en place une nouvelle méthode d'évaluation des transferts des métaux (SET) intégrant tous les mécanismes modulant la biodisponibilité grâce à des mesures biologiques. Le calcul d'un indice d'excès global de transfert de métaux (indice SET) permet d'identifier les transferts de métaux ainsi que leur intensité et lister les sites selon leur priorité de gestion. L'influence des paramètres du sol ayant été mise en évidence sur l'accumulation de métaux après 28 jours d'exposition, nous avons décidé, pour affiner la caractérisation de la biodisponibilité des métaux pour l'escargot, d'étudier les cinétiques d'accumulation sur trois sites industriels fortement contaminés en métaux du programme Bioindicateurs 2 (Metaleurop, SHSE et Auzon). La disparité de l'influence des paramètres des sols sur la biodisponibilité des métaux (estimée grâce aux flux d'assimilation) entre une exposition au laboratoire (influence très forte du pH, (Pauget et al., 2011)) et une exposition *in situ* (influence de la CEC, du taux de carbone organique et des limons, Tableau 14) nous a incité à réaliser deux expérimentations complémentaires en laboratoire pour tenter d'affiner et de mieux comprendre les mécanismes sous-jacents à la biodisponibilité des métaux. C'est dans cette optique que l'influence des paramètres édaphique sur la biodisponibilité du Cd, du Pb et du Zn a été étudiée à partir de sols contaminés en provenance du site de Metaleurop. Puis nous avons cherché à prédire la biodisponibilité du Cd, du Pb et du Zn par des extractants chimiques (estimateurs de la disponibilité environnementale) afin d'étudier la possibilité de s'affranchir des mesures biologiques et pour identifier les différentes fractions du sol dans lesquelles les métaux sont disponibles pour l'escargot. Les escargots étant exposés *in situ* à plusieurs sources de contamination (sol, végétation…), les contributions de la source sol et de la source végétation ainsi que l'influence des paramètres des sols sur ces contributions ont été quantifiées pour améliorer notre compréhension de la biodisponibilité du Cd et du Pb en termes de flux

Nous confronterons ici les résultats obtenus, en dégageant les principales avancées scientifiques de la thèse et les limites des réponses apportées. Pour formaliser les retombées de ces travaux en terme d'application à l'évaluation des risques un partie de cette discussion tentera de proposer un guide de choix des méthodes applicables selon la question posée par l'évaluateur de risque.

I.1. Apports de la thèse pour la compréhension des paramètres influençant l'accumulation et la biodisponibilité métaux pour l'escargot

I.1.a. Comparaison des influences des paramètres des sols in situ et au laboratoire sur la biodisponibilité des métaux

L'analyse des cinétiques d'accumulation *in situ* et en laboratoire révèle des différences quant à l'influence des paramètres du sol sur la biodisponibilité des métaux pour l'escargot (Figure 46). En effet, sur le terrain, une augmentation de la **CEC** provoque une diminution de la biodisponibilité du Cd tandis qu'elle augmente celle du Pb (Partie B, chapitre 3, Tableau 14). Cette augmentation de la biodisponibilité du Pb n'était pas attendue car une augmentation des sites de fixations des métaux dans les sols auraient normalement dû diminuer leur mobilité et donc leur biodisponibilité (Sterckeman et al., 2004). Cette diminution de la biodisponibilité sous l'influence de la CEC avait pourtant été mise en évidence pour les 2 métaux lors de l'expérimentation au laboratoire (Partie C, Chapitre 2, Tableau 24). Une hypothèse pourrait être que la CEC était expliquée à 79% par la teneur en argile des sols (Figure 44), donc la biodisponibilité du Pb serait plus importante sur les sols riches en argile. Bien que les argiles n'aient pas toutes la même capacité de rétention et de fixation des métaux (Bhattacharyya and Gupta, 2008), elles réduisent la mobilité des métaux tout comme la MO (Sterckeman et al., 2004). Cependant, nous avons observé qu'une augmentation de MO dans les sols faisait augmenter la biodisponibilité du Pb de par l'augmentation de la consommation de sol (Figure 46). L'augmentation de la biodisponibilité du Pb en relation avec l'augmentation de CEC (et incidemment d'argile) des sols pourrait être due à une augmentation de la consommation de sol par les escargots. En effet, les escargots se nourrissent de sol pour trouver des nutriments essentiels à leur développement (Gomot et al., 1989), un fort taux d'argile tout comme un fort taux de MO dans le sol pourrait jouer un rôle diluant forçant alors les escargots à consommer plus de sol pour satisfaire leur besoin nutritionnels augmentant ainsi leur contamination par la source sol.

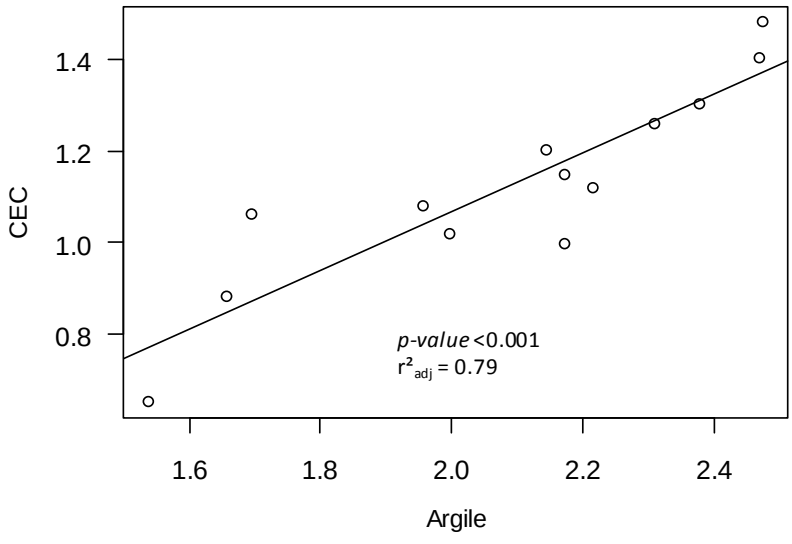


Figure 44 : Relation ente la CEC et le taux d'argile dans les sols des sites industriels d'Auzon, SHSE et de Metaleurop.

Tableau 28 : Equations des régressions multivariées mettant en relation les concentrations en Cd, Pb, As, Cr, Cu et Zn dans les viscères des escargots exposés aux modalités du programme Bioindicateurs sur lesquelles les végétaux ont été prélevés (n=25) pendant 28 jours avec (i) les concentrations totales en métal du sol, (ii) concentrations totales en métal du sol couplées aux paramètres physico-chimiques des sols (param.), (iii) les concentrations en métal dans les plantes, (iv) les concentrations totales en métal du sol couplées aux concentrations en métal dans les plantes et (v) les concentrations totales en métal du sol couplées aux concentrations en métal dans les plantes de la modalité et aux paramètres physico-chimiques des sols. ns : non significatif, ° p-value<0.1, *p-value<0.05, ** p-value<0.01, *** p-value<0.001

Métal	Régression	Equation de régression	*p-value*	r^2adj
Cadmium	Cd_{esc}~Cd_{sol}	$\log(Cd_{esc}+1) = 0.323^{***} + 0.396 \log(Cd_{sol}+1)^{***}$	<0.001	0.44
	Cd_{esc}~Cd_{vgx}	$\log(Cd_{esc}+1) = 0.273^{***} + 1.68 \log(Cd_{vgx}+1)^{**}$	<0.001	0.61
	Cd_{esc}~Cd_{sol}+param.	$\log(Cd_{esc}+1) = 0.761^{***} + 0.405 \log(Cd_{sol}+1)^{***} - 0.279 \log(C_{org}+1)^{*}$	<0.001	0.54
	Cd_{esc}~Cd_{sol}+Cd_{vgx}+param.	$\log(Cd_{esc}+1) = 0.543^{**} + 0.242 \log(Cd_{sol}+1)^{**} + 1.15 \log(Cd_{vgx}+1)^{***} - 0.193 \log(C_{org}+1)^{*}$	<0.001	0.75
Plomb	Pb_{esc}~Pb_{sol}	$\log(Pb_{esc}+1) = 0.223 + 0.367 \log(Pb_{sol}+1)^{***}$	0.001	0.37
	Pb_{esc}~Pb_{vgx}	$\log(Pb_{esc}+1) = 0.580^{***} + 2.015 \log(Pb_{vgx}+1)^{***}$	<0.001	0.52
	Pb_{esc}~Pb_{sol}+param.	$\log(Pb_{esc}+1) = 0.005^{ns} + 0.280 \log(Pb_{sol}+1)^{*} - 0.425 \log(C_{org}+1)^{\circ} + 25.5 \log(Fe_{ox}+1)^{**} + 0.133\ pH^{*}$	<0.001	0.61
	Pb_{esc}~Cc_{sol}+Pb_{vgx}+param.	$\log(Pb_{esc}+1) = -0.540^{*} + 1.68 \log(Pb_{vgx}+1)^{***} + 0.171\ pH^{***} + 12.1 \log(Fe_{ox}+1)^{*}$	<0.001	0.82
Arsenic	As_{esc}~As_{sol}	$\log(As_{esc}+1) = -0.110^{*} + 0.153 \log(As_{sol}+1)^{***}$	<0.001	0.63
	As_{esc}~As_{vgx}	$\log(As_{esc}+1) = 0.073^{\circ} + 0.316 \log(As_{vgx}+1)^{*}$	0.014	0.22
	As_{esc}~As_{sol}+param.	$\log(As_{esc}+1) = -0.110^{*} + 0.153 \log(As_{sol}+1)^{***}$	<0.001	0.63
	As_{esc}~Cc_{sol}+As_{vgx}+param.	$\log(As_{esc}+1) = -0.110^{*} + 0.153 \log(As_{sol}+1)^{***}$	<0.001	0.63
Chrome	Cr_{esc}~Cr_{sol}	$\log(Cr_{esc}+1) = -0.005 + 0.137 \log(Cr_{sol}+1)^{**}$	0.002	0.33
	Cr_{esc}~Cr_{vgx}	$\log(Cr_{esc}+1) = 0.092^{*} + 0.597 \log(Cr_{vgx}+1)^{***}$	<0.001	0.45
	Cr_{esc}~Cr_{sol}+param.	$\log(Cr_{esc}+1) = 0.181^{ns} + 0.122 \log(Cr_{sol}+1)^{***} + 0.151 \log(C_{org}+1)^{*} - 0.382 \log(CEC)^{***}$	<0.001	0.67
	Cr_{esc}~Cc_{sol}+Cr_{vgx}+param.	$\log(Cr_{esc}+1) = 0.181^{ns} + 0.122 \log(Cr_{sol}+1)^{***} + 0.151 \log(C_{org}+1)^{*} - 0.382 \log(CEC)^{***}$	<0.001	0.67
Cuivre	Cu_{esc}~Cu_{sol}	$\log(Cu_{esc}+1) = 1.92^{***} + 0.075 \log(Cu_{sol}+1)$	0.049	0.13
	Cu_{esc}~Cu_{vgx}	$\log(Cu_{esc}+1) = 1.41^{***} + 0.626 \log(Cu_{vgx}+1)$	0.05	0.13
	Cu_{esc}~Cu_{sol}+param.	$\log(Cu_{esc}+1) = 2.11^{***} + 0.011 \log(Cu_{sol}+1)^{ns} + 0.443 \log(CEC+1)^{***} - 0.220 \log(Limon+1)^{**}$	<0.001	0.56
	Cu_{esc}~Cc_{sol}+Cu_{vgx}+param.	$\log(Cu_{esc}+1) = 2.11^{***} + 0.011 \log(Cu_{sol}+1)^{ns} + 0.443 \log(CEC+1)^{***} - 0.220 \log(Limon+1)^{**}$	<0.002	0.56
Zinc	Zn_{esc}~Zn_{sol}	$\log(Zn_{esc}+1) = 2.81^{***} + 0.093 \log(Zn_{sol}+1)^{*}$	0.04	0.15
	Zn_{esc}~Zn_{vgx}	$\log(Zn_{esc}+1) = 2.11^{***} + 0.527 \log(Zn_{vgx}+1)^{**}$	0.005	0.29
	Zn_{esc}~ Zn_{sol} +param.	$\log(Zn_{esc}+1) = 3.11^{***} + 0.273 \log(Zn_{sol}+1)^{**} - 0.063\ pH^{\circ} - 0.289 \log(CEC+1)^{*}$	0.021	0.30
	Zn_{esc}~ Zn_{sol} +Zn_{vgx}+param.	$\log(Zn_{esc}+1) = 2.44^{***} + 0.048 \log(Zn_{sol}+1)^{ns} + 0.523 \log(Zn_{vgx}+1)^{**} - 0.204 \log(Argile+1)^{*}$	0.002	0.46

Tableau 29 : Equations des régressions multivariées mettant en relation les flux d'assimilation du Cd, Pb et As dans les viscères des escargots exposés aux modalités des sites SHSE, d'Auzon, et de Metaleurop du programme Bioindicateurs pendant 28 jours avec (i) les concentrations totales en métal du sol, (ii) les concentrations totales en métal du sol couplées aux paramètres physico-chimiques des sols (param.), (iii) les concentrations en métaux dans les plantes (iv) les concentrations totales en métal du sol couplées aux paramètres physico-chimiques des sols et aux concentrations en métaux dans les plantes de la modalité. ns : non significatif, ° p-value<0.1, *p-value<0.05, ** p-value<0.01, *** p-value<0.001

Métal	Régression	Equation de régression	*pvalue*	r^2_{adj}
Cd	a~Cd_{sol}	$\log(a_{Cd}+1) = ns$		
	a~Cd_{vgx}	$\log(a_{Cd}+1) = 0.019^{ns} + 0.223 \log(Cd_{vgx}+1)$**	0.001	0.64
	a~Cd_{sol}+param	$\log(a_{Cd}+1) = -0.336$** $+0.036 \log(Cd_{sol}+1)$* $- 0.218 \log(CEC+1)$* $+ 0.255 \log(Limons+1)$**	0.002	0.71
	a~Cd_{sol}+Cd_{vgx}+param	$\log(a_{Cd}+1) = -0.336$** $+0.036 \log(Cd_{sol}+1)$* $- 0.218 \log(CEC+1)$* $+ 0.255 \log(Limons+1)$**	0.002	0.71
Pb	a~Pb_{sol}	$\log(a_{Pb}+1) = ns$		
	a~Pb_{vgx}	$\log(a_{Pb}+1) = 0.008^{ns} + 1.19 \log(Pb_{vgx}+1)$*	0.013	0.42
	a~Pb_{sol}+param	$\log(a_{Pb}+1) = 1.59$* $+ 0.182 \log(Pb_{sol}+1)$* $+ 0.981 \log(CEC+1)$** $- 1.82 \log(C_{org}+1)$**	0.009	0.65
	a~Pb_{sol}+Pb_{vgx}+param	$\log(a_{Pb}+1) = 1.42$* $+ 1.38 \log(Pb_{vgx}+1)$** $- 0.939 \log(C_{org}+1)$*	0.002	0.70
As	a~As_{sol}	$\log(a_{As}+1) = ns$		
	a~As_{vgx}	$\log(a_{As}+1) = ns$		
	a~As_{sol}+param	$\log(a_{As}+1) = ns$		
	a~As_{sol}+As_{vgx}+param	$\log(a_{As}+1) = ns$		

Concernant les autres paramètres des sols, nous avons vu qu'en laboratoire, nous avions identifié une influence nette du **pH et des oxydes de fer et d'aluminium** alors que lors de l'exposition des escargots sur le terrain aucun de ces paramètres n'a pu être identifié (Figure 46). L'absence d'influence des paramètres des sols sur la biodisponibilité et l'accumulation des métaux chez l'escargot a également pu être mise en évidence lors d'une expérimentation en bioindication passive d'escargots de la même espèce sur des sols du site de Metaleurop (Mourier et al., 2011).
Toutes ces différences montrent que l'extrapolation de données issues d'expérimentation en laboratoire pour expliquer la biodisponibilité des métaux *in situ* est délicate car on ne considère pas les interactions possibles entre les différents facteurs (plusieurs sources, mobilité des métaux, compétition des métaux sur les sites de fixation…).

I.1.b. Influence des caractéristiques des sols et des sources de contamination sur l'accumulation et la biodisponibilité des métaux

Suite à la mise en évidence de l'influence de la matière organique et du pH sur la biodisponibilité des métaux du sol pour l'escargot (Pauget et al., 2011), nous avons cherché à voir si cette influence était identifiable *in situ* (Partie B, Chapitre 2). L'utilisation de régressions multivariées a révélé que de forts taux de carbone organique limitaient l'accumulation du Cd, du Pb et du Zn tandis que les oxydes de fer et d'aluminium auraient tendance à faciliter l'accumulation du Pb et du Zn (respectivement). Cependant, même si l'influence de ces paramètres sur l'accumulation est nette et significative, l'accumulation des métaux n'est expliquée au maximum qu'à 56% pour le Pb par la concentration totale du sol et certaines de ses caractéristiques physico-chimiques (Tableau 10), laissant supposer que d'autres paramètres interviennent dans la modulation de la biodisponibilité. L'influence des sources de contamination ainsi que des paramètres des sols ayant été mise en évidence sur l'accumulation après 28 jours d'exposition, il était donc nécessaire de caractériser ces influences sur les flux et les transferts de métaux, c'est pourquoi nous avons réalisé trois études cinétiques.
La première (Partie B, Chapitre 3), *in situ* sur trois sites industriels du programme Bioindicateurs 2 (Auzon, Metaleurop et SHSE), a permis de caractériser la biodisponibilité des métaux pour l'escargot ainsi que de mettre en évidence **l'influence des paramètres des sols** sur la biodisponibilité des métaux. Cette étude a également soulevé la question de l'influence des sources de contamination sur la biodisponibilité des métaux et dans quelle mesure les paramètres des sols modulent la contribution et la biodisponibilité des métaux de chaque source.
Les deux études suivantes visaient donc à répondre à ces questions. Réalisées en laboratoire elles ont permis pour la première de quantifier la contribution de la source sol et de la source plante à la biodisponibilité des métaux ainsi que d'estimer l'influence des paramètres du sol sur cette contribution (Partie C, Chapitre 1) et pour la seconde de déterminer l'influence des paramètres des sols sur des sols contaminés du site de Metaleurop (Partie C, Chapitre 2). Cette seconde étude en laboratoire ayant pour seule source de contamination le sol nous a permis de nous affranchir de l'influence des sources de contamination sur la biodisponibilité des métaux et de déterminer les pools disponibles du sol du Cd, du Pb et du Zn pour l'escargot.
L'influence des sources de contamination sur la biodisponibilité des métaux ayant été mise en évidence en laboratoire, il nous est apparu intéressant de voir si l'ajout des concentrations en métaux des plantes (données acquises par une autre équipe (Faure et al., 2011) sur les modalités boisées (n=25) du programme Bioindicateurs 2) comme variable explicative permettaient d'améliorer

nos modèles prédictifs *in situ*. Nous avons développé de nouvelles équations mettant en relation l'accumulation et l'assimilation (a) de métaux dans les escargots avec (i) les concentrations totales en métal du sol puis (ii) les concentrations en métaux dans le végétaux, puis nous avons identifié l'influence des paramètres du sol sur l'accumulation et la biodisponibilité des métaux. Pour finir, nous avons cherché à déterminer des équations prédictives de l'accumulation et des flux d'assimilation des métaux en couplant concentration en métal dans le sol et dans les plantes et caractéristiques physico-chimiques des sols (Tableau 28 pour l'accumulation et Tableau 29 pour l'assimilation).

NB : les valeurs de concentrations dans les plantes utilisées correspondent à la médiane des concentrations en métaux de plusieurs espèces échantillonnées sur l'ensemble de chaque modalité et non au sein de chaque microcosmes ce qui constitue un facteur d'imprécision. L'ajout des concentrations en métaux dans les plantes comme variable explicative a permis de mieux expliquer l'accumulation du Cd et du Zn dans les escargots. En effet, les estimations de l'accumulation de ces deux métaux estimées grâce à la concentration totale et aux paramètres des sols sont améliorées de 21 et 16% (pour le Cd et le Zn, respectivement) en ajoutant la concentration en métaux dans les plantes. De plus, dans le Tableau 29, nous pouvons voir que la biodisponibilité du Cd, qui était expliquée par la concentration totale du Cd dans les sols couplée aux paramètres des sols à 71%, est expliquée à 64% par la concentration en Cd dans les végétaux. La concentration en Cd dans les végétaux serait donc un bon indicateur de la biodisponibilité du Cd pour les escargots (et inversement). L'influence significative de la source plante sur l'accumulation et la biodisponibilité du Cd chez *C. aspersus* a déjà été démontrée auparavant (Scheifler et al., 2006) lors d'une expérimentation en laboratoire avec de la laitue.

Pour le Pb, de manière surprenante, la concentration en métaux dans les plantes se révèle être un meilleur indicateur de son accumulation dans les viscères des escargots que sa teneur dans le sol (r^2_{adj} = 0.52 contre 0.37, Tableau 28). De plus, la concentration dans les plantes couplée aux paramètres des sols permettent d'expliquer 82% de la variation de son accumulation tandis que la concentration en Pb du sol couplée aux paramètres des sols ne permet d'expliquer que 61% de cette variation. Cependant, sa concentration dans les plantes est elle-même expliquée à 82% par la teneur en Pb dans le sol couplée aux paramètres des sols (donnée non montrée). De même, l'ajout de la concentration en Pb dans les végétaux permet d'améliorer l'explication de la variation de sa biodisponibilité pour l'escargot de 65% à 70%, les flux d'assimilation étant corrélés à la concentration en Pb dans les végétaux à 42% (Tableau 29). L'ajout de la concentration en métaux dans les végétaux masque l'influence de la CEC et de la concentration en Pb du sol précédemment identifiée (Tableau 29). La disparition de ces deux paramètres permettant d'expliquer la variabilité de la biodisponibilité est due en grande partie à leur corrélation avec la concentration en Pb dans les végétaux. La concentration totale en Pb, le C_{org} et l'argile (ces deux derniers conditionnant la CEC) expliquent 86% des variations de concentration en Pb dans les végétaux (données non montrées). La concentration en Pb dans les végétaux serait donc un bon estimateur de l'accumulation et de la biodisponibilité du Pb *in situ* par l'escargot comme montré par leur bonne corrélation (r^2_{adj}= 0.52 pour l'accumulation, Tableau 28 et r^2_{adj}= 0.42 pour la biodisponibilité, Tableau 29). Ces corrélations ne remettent pas en question le fait que la source principale du Pb biodisponible et accumulé par les escargots soit le sol. Elles peuvent montrer une similitude entre l'accumulation par les escargots et par les végétaux de ce métal. Il est également possible qu'*in situ* le pool de métaux disponibles soit différent de celui déterminé au laboratoire (conditions expérimentales modifiant la répartition des métaux entre les phases solide et liquide). Cependant, il n'est pas exclu que la contribution des végétaux à

l'accumulation et à la biodisponibilité du Pb dans les viscères joue un rôle plus important que celui mesuré en laboratoire, la présence de poacées ou d'orties (espèces hyperaccumulatrices de métaux) sur le site rendant l'extrapolation des données obtenues avec de la laitue difficile. De plus, comme souligné précédemment, les concentrations en métaux *in situ* ont été mesurées dans des plantes et dans des sols échantillonnés sur l'ensemble de la parcelle et ne sont pas forcément représentatives de ce qui se trouvait précisément dans les microcosmes.

Pour l'As et le Cu, les concentrations en métaux dans les plantes ne permettent par d'améliorer l'estimation des concentrations accumulées de ces deux métaux (Tableau 28). La concentration en As et Cu des plantes, contrairement à celle en Cd et Pb, n'est donc pas un bon estimateur de l'accumulation de l'As et du Cu par l'escargot. Pour l'As, aucun paramètre du sol n'ayant été identifié comme modulant son accumulation ainsi que sa biodisponibilité (Tableau 29), nous supposons que le paramètre principal qui module son accumulation et sa biodisponibilité est sa valence dans les sols qui modifie sa mobilité déjà reconnue comme faible (INERIS, 2010).

Pour le Cr, même si l'équation prédictive de son accumulation dans les escargots n'est pas améliorée par la prise en compte des concentrations en métaux dans les végétaux, on remarque une meilleure corrélation entre l'accumulation de Cr et ces dernières et qu'avec la concentration totale en Cr du sol. Cependant, il apparait que les concentrations en Cr dans les plantes et dans les sols sont corrélées (données non montrée) ce qui les rend quasiment interchangeables. En effet, la concentration en Cr dans les plantes couplée aux paramètres des sols permet d'expliquer 62% de la variation de l'accumulation du Cr chez l'escargot (équation non montrée) contre 67% si on utilise la concentration totale dans le sol couplée aux paramètres physico-chimiques.

La prise en compte de la contamination en métaux du sol, de la concentration en métaux des plantes et des caractéristiques du sol permet d'expliquer 46 à 82% de l'accumulation et de la biodisponibilité des métaux étudiés pour les escargots, il apparait donc nécessaire d'inclure ces paramètres modulant l'accumulation et la biodisponibilité des métaux lors d'ERE. Pour des approches prédictives, il reste néanmoins à améliorer encore ces équations notamment pas la prise en compte d'autres facteurs tels que les contributions des sources de contamination qui peuvent grandement moduler la le pool biodisponible de métaux, l'occupation des sols dont l'influence sur le transfert a déjà été démontrée (Fritsch, 2010) ainsi que le climat modulant l'activité des escargots sentinelles…

I.2. Apports de la thèse pour l'évaluation des risques résultants de la contamination des sols

I.2.a. Estimation de la biodisponibilité par des méthodes chimiques

Aujourd'hui de nombreuses études ont porté sur la mise en place de mesures chimiques de la biodisponibilité des métaux pour différents organismes (Peijnenburg et al., 2007; ISO 17402, 2008; Roulier et al., 2008; Smith et al., 2010; Mourier et al., 2011; Pauget et al., 2011). Cependant, à l'heure actuelle le nombre de méthodes chimiques validées pour divers couple (métal-organismes reste limité (van Gestel, 2008). Lors d'une expérimentation préliminaire, nous avions mis en évidence grâce à l'utilisation des taux d'absorption (k_1), que ni la concentration totale ni la concentration extraite au $CaCl_2$ ne permettait d'estimer la biodisponibilité du Cd et du Pb (Pauget et al., 2011). Cependant, cette étude a été réalisée sur des sols artificiellement contaminés et donc peu représentatifs de la réalité. C'est pour cette raison que nous avons cherché à étudier la capacité de sept méthodes chimiques d'estimation de la disponibilité environnementale (*i.e.* fraction des métaux supposée biodisponible) à prédire la biodisponibilité du Cd, du Pb et du Zn de sols issus d'un site

contaminés. Cette méthode nous a permis de mettre en évidence que la biodisponibilité (*i.e.* flux d'assimilation, a) du Cd et du Pb pour l'escargot pouvait être estimée respectivement par la concentration extraite à l'EDTA et par la concentration totale alors que pour le Zn, aucune ne le permettait. Nous en avons donc conclu qu'il n'existait pas de méthode générale d'estimation de la biodisponibilité et, qu'en accord avec van Gestel (van Gestel, 2008), des mesures de la biodisponibilité réelle par des bioindicateurs sont encore nécessaires non seulement pour les métaux déjà étudiés mais également pour les nombreux contaminants pour lesquels les extractants n'ont pas encore été testés.

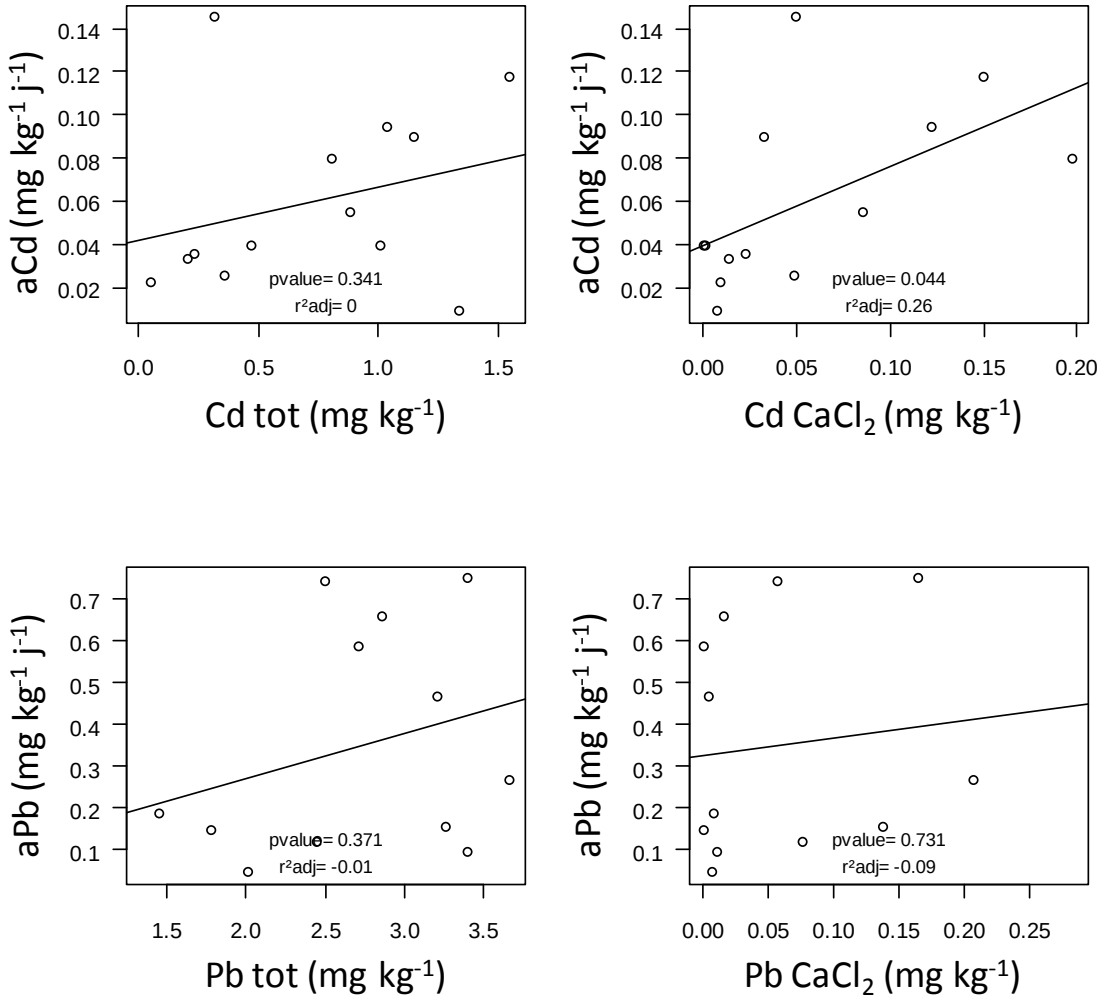


Figure 45 : Relation entre les flux d'assimilation *in situ* (a) du Cd (haut) et du Pb (bas) et la concentration totale du sol (gauche) et la concertation extraite au $CaCl_2$ (droite).

Dans le cadre du programme Bioindicateurs 2, nous disposions des concentrations extraites au $CaCl_2$ dans les sols, nous avons donc cherché à voir si la concentration totale ou la concentration extraite au $CaCl_2$ du sol permettait de prédire la biodisponibilité des métaux (Figure 45). L'absence de relation entre flux d'assimilation et concentration totale (Figure 45) nous permet de dire que la concentration totale n'est pas un bon estimateur de la biodisponibilité du Cd et du Pb *in situ* pour l'escargot contrairement à ce qui avait déterminé en laboratoire lorsque le sol était la seule source de contamination (Partie C, Chapitre 2, Tableau 26). Les différentes sources de contamination telles que

le sol, les végétaux (etc.) ainsi que les différences d'exposition précédemment soulevées jouent un rôle très important dans la biodisponibilité *in situ* des métaux renforçant ainsi l'idée que mesurer uniquement les concentrations totales en métaux d'un sol lors de procédures d'évaluation des risques est insuffisant. Au cours de l'exposition sur le terrain, nous avons pu mettre en évidence que le $CaCl_2$ permettait d'expliquer à 26% les variations des flux d'assimilation du Cd mais pas celles des flux d'assimilation du Pb (Figure 45). Cette corrélation entre biodisponibilité du Cd et concentration extractible au $CaCl_2$ peut être expliquée par la présence de végétaux contaminés durant l'étude *in situ*., Sachant que les concentrations en métaux dans les végétaux peuvent être estimées par le $CaCl_2$ (Meers et al., 2007a) et que les végétaux sont la principale source de Cd pour les escargots, la corrélation entre biodisponibilité du Cd et concentration extraite au $CaCl_2$ représenterait donc la corrélation entre la biodisponibilité du Cd et celle des plantes.

I.2.b. Evaluation du transfert de métaux in situ : l'indice SET

La multitude de paramètres à prendre en compte pour prédire l'accumulation et la biodisponibilité des métaux pour l'escargot nous a donc conduits à l'élaboration d'une méthodologie intégrative de ces paramètres pour évaluer des transferts de métaux supérieurs à la normale : l'indice SET. Cette méthode, basée sur une mesure biologique globale des transferts de métaux aux escargots exposés au sein d'un site à toutes les sources de contamination et aux aléas climatiques, permet de visualiser la biodisponibilité des métaux en intégrant tous les paramètres qui la module. Cette méthodologie permet de caractériser les transferts de métaux dans le but de hiérarchiser les sites selon leur risque global de transfert de cinq métaux (trois non essentiels et deux essentiels) et d'un métalloïde (Figure 46). Elle a permis de mettre par exemple en évidence une absence de transfert sur un site industriel bien que ses sols soient contaminés en plusieurs métaux notamment le Cd, le Pb et l'As. L'établissement des CIRef constitue un nouvel outil présentant de multiples intérêts pour les études d'évaluation des risques. En effet, cette mesure biologique du risque intègre tous les paramètres physico-chimiques et biologiques qui peuvent influencer le transfert et permet de proposer un référentiel à l'échelle nationale (France) des transferts de métaux aux escargots.

II. Conclusion

II.1. Apport de la thèse à l'ERE

Au début de cette thèse, de nombreux manques ont été soulevés comme l'absence de prise en compte des paramètres des sols dans les études d'évaluation des risques, le manque de référentiel de concentration interne des escargots utilisés en bioindication active…

Les études que nous avons menées tant *in situ* qu'au laboratoire nous ont permis de fournir des informations sur la biodisponibilité des métaux ainsi que des éléments de compréhension des mécanismes qui régissent la biodisponibilité des métaux pour l'escargot *C. aspersus*.

Même si l'escargot est couramment utilisé en bioindication (Gomot de Vaufleury and Pihan, 2000; Regoli et al., 2006; Gimbert et al., 2008a; Coeurdassier et al., 2010), l'étude de l'accumulation de métaux sur un large panel de sites contaminés et non contaminés n'avait jamais été réalisée. Cette étude nous a permis de déterminer des concentrations internes de référence (CIRef) pour le Cd, le Pb, l'As, le Cr, le Cu et le Zn grâce à l'exposition d'escargot sur les sites non contaminé en métaux (Figure 46, 1). Ces CIRef sont la pierre angulaire de la méthodologie SET (Somme des Excès de Transfert) développée au cours de cette thèse. Cet outil proposant des notes représentatives du risque de transfert global de métaux (SET_{plot} et SET_{site}) permettra de répondre aux attentes des

politiques publiques quant au développement d'outils permettant d'identifier les risques liés aux métaux. De plus cette méthodologie basée sur une mesure réelle de la biodisponibilité des métaux et non plus sur les concentrations totales des métaux dans les sols permettra de quantifier l'efficacité des méthodes de gestion des sites et sols pollués par comparaison des notes de SET avant et après remédiation.

L'étude des cinétiques d'accumulation *in situ*, a permis de renseigner pour la première fois la biodisponibilité de l'As et du Sb via les flux d'assimilation et les taux d'excrétion. Cette étude met en avant la faible biodisponibilité de ces deux métalloïdes sur des sols pourtant fortement contaminés. L'absence de corrélation entre leur flux d'assimilation avec la concentration totale indique que les escargots n'ont pas accès à la totalité de ces contaminants dans les sols. De plus, l'absence d'influence des paramètres des sols sur la modulation de la biodisponibilité indique qu'une analyse plus fine des constituants du sol (matière organique en suspension /particulaire…) doit être réalisée et/ou que d'autres paramètres doivent être pris en considération comme la nature des argiles ou la spéciation des métaux dans les sols (Figure 46, 2). En effet, la mobilité de ces deux métalloïdes varie en fonction de leur forme cationique dans les sols (Wilson et al., 2010). Cette étude souligne également l'intérêt de l'utilisation des cinétiques d'accumulation lors d'études de la biodisponibilité car les concentrations à l'équilibre n'ont pu être atteintes durant la période d'exposition de 28 jours risquant ainsi de sous estimer les facteurs de bioaccumulation.

Dans le but de mieux comprendre les mécanismes qui modulent la biodisponibilité du Cd, du Pb, de l'As et du Sb, l'influence des paramètres des sols a été étudiée *in situ*. Les différences observées entre labo et terrain (Figure 46, 2) résultent de la variabilité de l'exposition (condition climatiques, sources de contamination…) et que le choix des approches (terrain ou laboratoire) doit petre réfléchit selon les questions posées (Tableau 30). De la mise en relation de l'accumulation et de la biodisponibilité des métaux avec la concentration en métaux dans les plantes, nous avons tiré que la contamination des plantes était importante à prendre en considération pour certains métaux lors d'études de la biodisponibilité *in situ* bien que les concentrations en métaux et les caractéristiques physico-chimiques des sol permettent déjà d'estimer de 41 à 67% de l'accumulation des métaux étudiés (Partie B, chapitre 2, Tableau 10).

Site	SET_{site}
Metaleurop	4.261
Auzon	3.206
SHSE	2.259
RENECOFOR	0.732
Qualiagro	0.018
GISFI	0.011
BioREco	0.003
Thil	0
Yvetot	0

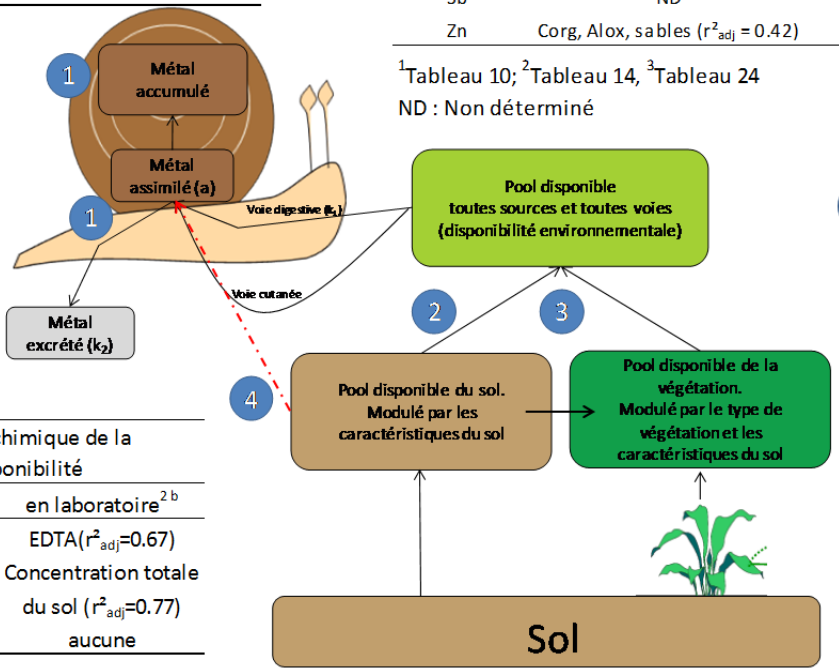
1

Métal	CIRef[a] ($mg.kg_{esc}^{-1}$)
As	0.307
Cd	2.27
Cr	2.01
Cu	185
Pb	12.9
Sb	ND
Zn	1490

2

Métal	caractéristique du sol modulant		
	l'accumulation (C28)	la biodisponibilité (a)	
	in situ [1 a]	*in situ* [2 a]	laboratoire[3 b]
As	aucune (r^2_{adj} = 0.67)	non significatif	ND
Cd	C_{org} (r^2_{adj} = 0.51)	CEC, limons (r^2_{adj} = 0.36)	pH, CEC, Fe_{ox} (r^2_{adj} = 0.81)
Cr	Corg, CEC, sables (r^2_{adj} = 0.41)	ND	ND
Cu	Argiles, Alox, limons (r^2_{adj} = 0.41)	ND	ND
Pb	pH, Feox, Corg (r^2_{adj} = 0.56)	CEC, C_{org} (r^2_{adj} = 0.71)	CEC (r^2_{adj} = 0.83)
Sb	ND	non significatif	ND
Zn	Corg, Alox, sables (r^2_{adj} = 0.42)	ND	pH, Al_{ox}, Fe_{ox} (r^2_{adj} = 0.75)

[1]Tableau 10; [2]Tableau 14, [3]Tableau 24
ND : Non déterminé



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Métal	Source	Contribution moyenne [a]	effet ↗ MO	effet ↘ pH
Cd	sol	31.90%	↗	↘
	Laitue	68.10%	↘	↗
Pb	sol	89.90%	↗	↘
	Laitue	10.10%	↘	↗

[a] : exposition multi-source
[b] : exposition mono-source (source sol)

4

Métal	estimateur chimique de la biodisponibilité	
	in situ [1 a]	en laboratoire[2 b]
Cd	$CaCl_2$ (r^2_{adj}=0.21)	EDTA(r^2_{adj}=0.67)
Pb	aucune	Concentration totale du sol (r^2_{adj}=0.77)
Zn	ND	aucune

[1]Tableau 15, [2]Tableau 26
ND : Non déterminée

Figure 46 : Bilan des réponses apportées au cours de cette thèse

Pour répondre aux besoins des politiques publiques quant au développement d'outils chimiques permettant d'estimer la biodisponibilité des métaux, nous avons testé la capacité du $CaCl_2$, méthode d'extraction recommandée par l'ISO (ISO 17402, 2008), à estimer la biodisponibilité du Cd et du Pb *in situ*. Nous avons pu observer que cette mesure chimique permettait d'estimer environ 20% de la variation du flux d'assimilation du Cd mais ne permettait pas d'expliquer les variations de la biodisponibilité du Pb. Il a été démontré en laboratoire (Partie C, Chapitre 2) que l'escargot est capable d'assimiler les métaux fortement liés à la phase solide du sol bien que que l'influence des paramètres des sols restait significative.

II.2. Aide à la sélection des outils utilisés au cours de la thèse pour répondre à diverses questions de l'ERE

Au cours de cette thèse, deux conditions expérimentales (terrain et laboratoire) ainsi que deux méthodes de mesures de la biodisponibilité (statique et cinétique) ont été utilisées. Cette partie vise à présenter les intérêts et les limites de chacune. Ces éléments constituant les bases pour choisir l'outil le plus adapté à la situation lors d'études d'ERE (Tableau 30).

II.2.a. Expérimentation in situ ou en laboratoire ?

Nous avons vu que la biodisponibilité était dépendante de nombreux paramètres et que leur prise en compte s'avère nécessaire pour l'ERE. Cependant, il s'avère également important de bien choisir les conditions expérimentales pour répondre le plus précisément à l'objectif fixé. En effet, pour caractériser la biodisponibilité des métaux pour un bioindicateur donné (voir même un couple métal-bioindicateur), il apparait qu'une mesure *in situ* soit la meilleure solution. En effet, sur le terrain, le bioindicateur est soumis à l'ensemble des paramètres modulant la biodisponibilité comme les paramètres des sols (modulant la mobilité des métaux), le climat (modulant l'activité du bioindicateur), les sources de contribution (biodisponibilité des métaux différentes d'une source à l'autre),… La mesure *in situ* apparait donc comme intégratrice et reflétant la biodisponibilité des métaux la plus représentative de la réalité. Cependant, si on souhaite comprendre les mécanismes modulant la biodisponibilité, une expérimentation *in situ* s'avère délicate car le grand nombre de paramètres agissant de concert rend l'interprétation difficile. Par contre, l'expérimentation au laboratoire permettra d'isoler le ou les paramètre(s) influençant la biodisponibilité et d'en étudier précisément ses mécanismes.

II.2.b. Expérimentation statique ou cinétique ?

Après avoir choisit son mode d'expérimentation (laboratoire ou terrain), il importe de choisir d'inclure ou non l'aspect dynamique de la biodisponibilité. Quelque soit le choix, la concentration en contaminant dans les escargots après 28 jours d'exposition (C_{28}) sera déterminée. En fonction des moyens (moyens financiers et matériels, disponibilité de personnel) il sera toujours préférable de choisir une expérimentation cinétique afin de prendre en compte les aspects dynamiques de la biodisponibilité, la vitesse d'entrée du métal dans l'organisme pouvant être une variable importante à connaitre pour comprendre l'effet d'un contaminant sur l'organisme. Elles permettent également d'identifier les flux de métaux dans l'organisme quand une mesure statique n'identifie aucune accumulation (Figure 47). De même, les cinétiques d'accumulation permettent de déterminer les concentrations à l'équilibre d'un contaminant, paramètre nécessaire pour une bonne ERE.

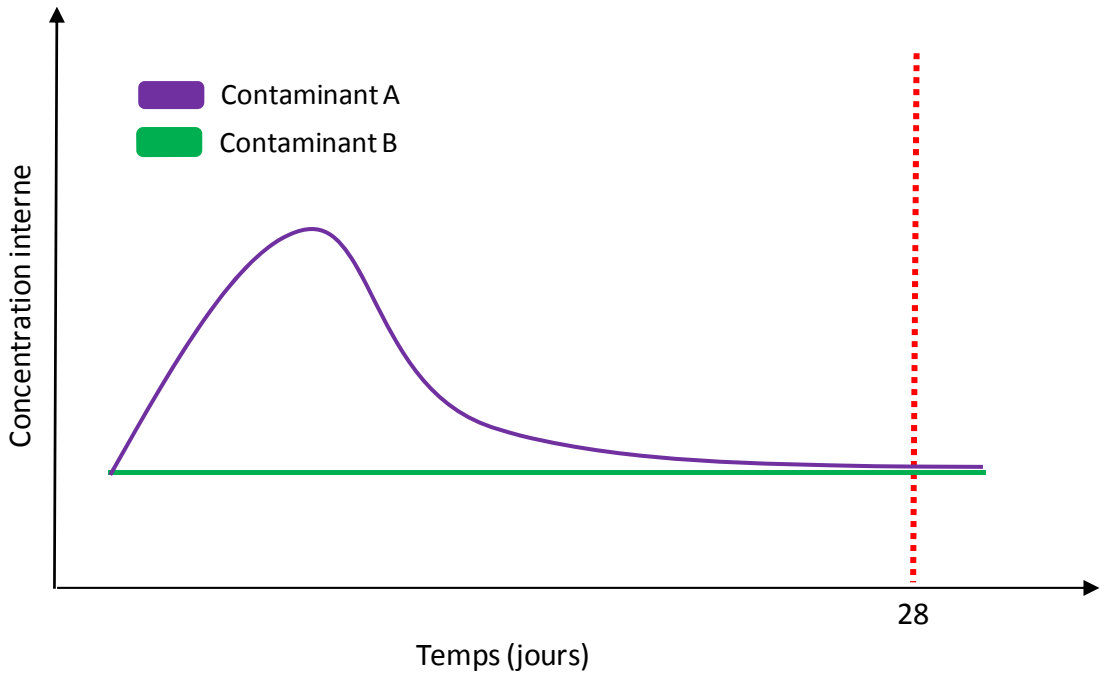

Figure 47 : Cinétique d'accumulation théorique de deux contaminants. La ligne rouge représente un échantillonnage après 28 jours d'exposition.

Cependant, même si les cinétiques d'accumulation sont plus informatives que les études statiques, ces dernières n'en restent pas moins très utiles pour effectuer un premier screening de la biodisponibilité et de l'accumulation des métaux dans les organismes. De plus, il est à noter que le pas de temps de prélèvement d'une étude cinétique doit être choisit avec précaution afin de ne pas passer à coté d'un flux entrant qui pourrait être masqué très rapidement par une forte excrétion. Il est donc primordial de focaliser l'effort d'échantillonnage sur les premiers jours voire les premières heures de l'exposition. Une fois de plus, l'effort d'échantillonnage dépendra des moyens à disposition de l'opérateur sachant que plus l'effort d'échantillonnage sera important plus la réponse pourra être précise.

Tableau 30 : Méthodes d'études et outils de caractérisation de l'accumulation et de la biodisponibilité : intérêts et limites.

		Intérêts	Limites
Méthode d'étude	Expérimentation *in situ*	Mesure réelle intégrant l'influence de tous les paramètres qui modulent la biodisponibilité	le grands nombre de paramètres modulant la biodisponibilité pris en même temps rend la quantification de leur influence très compliquée. Nécessite des déplacements, inclus des risques de vandalisme et de la prédation des bioindicateurs…
	Expérimentation en laboratoire	Permet d'étudier séparément tous les paramètres influençant la biodisponibilité et de quantifier leur influence	Rend une image partielle de la biodisponibilité car seul(s) le(s) facteur(s) étudié(s) sont pris en compte
	Etude statique	mesure ponctuelle, permet d'identifier une accumulation de métaux en une seule mesure	mesure ponctuelle, ne prend pas en compte les processus dynamiques de la biodisponibilité, ne procure aucune information sur les flux et états d'équilibre
	Etude cinétique	renseigne sur les processus dynamiques de la biodisponibilité ainsi que sur l'état d'équilibre du contaminant dans l'organisme. Permet la modélisation de paramètres comme le flux d'assimilation et le taux d'excrétion grâce aux patterns d'accumulation	Etude lourde au niveau logistique et plus couteuse qu'une étude statique
	Estimation Biodisponibilité par des méthodes chimiques (C_x)	Plus de mesures létales, identification de la biodisponibilité à moindre coût et rapidement, renseigne sur biodisponibilité des métaux des différentes phases du sol	les méthodes chimiques doivent intégrer l'ensemble des paramètres physico-chimiques et biologiques modulant la biodisponibilité, la méthode d'extraction chimique doit être déterminée pour un couple organisme-contaminant. A ce jour, aucune méthode chimique ne parait être pleinement fonctionnelle pour prédire la biodisponibilité pour l'escargot.
Endpoint	Concentration à 28 jours (C_{28})	Renseigne sur l'accumulation et la biodisponibilité d'un contaminant après un temps d'exposition donné	ne renseigne pas sur les flux de contaminant dans l'organisme
	SET (Somme des Excès de Transfert)	Mise en place de valeurs de référence pour l'escargot à l'échelle nationale intégratrice de tous les paramètres influençant la biodisponibilité, identifie et quantifie les excès de transfert pour 6 éléments, permet d'établir des priorités de gestion et quantifie l'efficacité des méthodes de réhabilitation d'un site	Ne permet pas l'identification et la quantification des paramètres modulant la biodisponibilité ainsi que l'état d'équilibre des éléments dans l'organisme
	Facteur de bioaccumulation statique ($FBA28 = C_{28}/C_{ext}$)	identifie l'accumulation à un instant donné	ne prenant pas en considération les processus dynamiques de la biodisponibilité, risque de sous- / surestimation si la concentration à l'équilibre dans l'organisme n'est pas atteinte. Ne prends pas en considération la concentration en métaux des autres sources de contamination
	Flux d'assimilation (a)	renseigne sur la vitesse d'entrée des polluants dans l'organisme toutes voies confondues en prenant en compte tous les paramètres physico-chimiques modulant la biodisponibilité	paramètre modélisé et non mesuré
	Taux d'excrétion (k_2)	permet de mettre en évidence une excrétion et permet le calcul de concentration à l'équilibre dans l'organisme et de FBA_{ss}	paramètre modélisé et non mesuré
	Concentration à l'équilibre ($C_{ss} = a/k_2$)	renseigne sur la concentration à l'équilibre dans l'organisme en ayant pris en compte les flux de métaux dans l'organisme	ne peut être calculée seulement si une excrétion significative est modélisée
	Taux d'absorption ($k_1 = a/C_x$)	renseigne sur l'exposition des organismes par la voie digestive et permet la comparaison de l'influence des paramètres du sol sur la disponibilité environnementale estimé par une méthode chimique et sur la biodisponibilité et permet le calcul de FBA_{ss}	Puisque calculé sur la base d'une concentration supposée biodisponible, la méthode chimique d'estimation du Cx doit être représentative du pool réellement disponible pour l'escargot. Ne prend pas en considération les autres sources de contamination
	Facteur de bioaccumulation à l'équilibre ($FBA_{ss} = k_1/k_2$)	Renseigne sur l'accumulation des métaux en intégrant les processus dynamique de la biodisponibilité	puisque basé sur le k1 présente les mêmes inconvénients. De plus quand l'excrétion n'est pas significative, cette valeur n'est pas calculable.

III. Perspectives

Les mécanismes modulant la biodisponibilité ont donc été explorés et en partie expliqués au cours de cette thèse. Cependant, il reste encore de nombreux paramètres influençant la biodisponibilité à prendre en compte afin de prédire de manière satisfaisante la biodisponibilité des métaux des sols lors d'études *in situ*.

III.1. Elargissement de l'indice SET

Le SET, déjà integrateur d'un risque général de transfert puisque basée sur l'étude de cinq métaux et un métalloïde (Cd, Pb, Cr, Cu, Zn et As), pourrait être complétée par la détermination de nouvelles CIRefs. En effet, plus l'outil SET s'appuiera sur une large gamme de concentrations de référence et plus cet outil sera intégrateur d'un risque global de transfert de métaux. Pour cela, il faudrait exposer d'autres individus aux sites non contaminés du programme Bioindicateurs. Ainsi, les CIRef d'autres métaux et métalloïdes comme le molybdène (Mo), le mercure (Hg), le cobalt (Co), le nickel (Ni), le thallium (Tl), l'antimoine (Sb), l'étain (Sn), le strontium (Sr) (etc.) pourraient être déterminées afin d'avoir un outil de gestion des sites intégrant la quasi-totalité des métaux recensés comme prioritaires pour la préservation de l'environnement et de la santé humaine. La prise en compte de ces métaux dans l'indice SET permettrait à un gestionnaire d'avoir une réponse encore plus complète quant au risque global de transfert des métaux de son site vers un organisme bioindicateur reconnu de la qualité des sols. De même, les CIRef de tous les polluants organiques pour lesquels très peu de données sont disponible pourraient être déterminées. Ainsi, l'augmentation du nombre de CIRef utilisées élargirait d'autant plus les possibilités d'utilisation de l'indice SET. Il serait également intéressant de mettre en application cette méthode sur un site nécessitant une réhabilitation et de comparer les notes de SET que ce site obtiendrait avant et après traitement.

III.2. Mise en relation des flux d'assimilation et des effets des métaux.

Au cours de cette thèse nous nous sommes focalisés sur les deux premiers volets de la biodisponibilité (*i.e.* disponibilité environnemental et biodisponibilité environnementale). Cependant, suite à la caractérisation de la biodisponibilité des métaux par les flux d'assimilation via les cinétiques d'accumulation, il serait intéressant d'étudier la possible corrélation entre vitesse d'entrée des métaux dans l'organisme et apparition d'effets délétères. Parmi le grand nombre de méthodes utilisées pour identifier les effets des métaux, la quantification des cassures des brins d'ADN par le test comète (= Single Cell Gel Electrophoresis (SCGE)) paraissent prometteuses (Button et al., 2010; Itziou et al., 2011). Afin de mettre en évidence la relation entre dommage cellulaire et flux d'assimilation des cinétiques d'accumulation pourraient être réalisées sur des sols contaminés. Pour chaque escargot échantillonné, un volume d'hémolymphe serait prélevé en vue d'effectuer le test comète sur les cellules circulantes. Le pourcentage de cassure d'ADN déterminé grâce à la densité de la queue serait alors mis en relation avec la vitesse d'entrée du métal dans l'organisme. L'établissement d'une bonne corrélation entre ces deux paramètres pourrait alors servir de pont pour prédire des effets grâce aux concentrations en contaminant et aux caractéristiques physico-chimiques des sols pour plus tard tenter de relier un effet génotoxique à un effet de niveau supérieur (niveau individuel ou populationnel).

III.3. Détermination de la contribution des sources de contamination in situ

Afin d'améliorer les estimations de la biodisponibilité, il serait intéressant d'étudier les contributions de chaque source de contamination *in situ*. En effet, cette contribution pourrait être variable en fonction du cortège floristique présent et ingéré par l'escargot. Il serait donc intéressant de réaliser une étude cinétique d'accumulation des métaux *in situ* en microcosme en exposant des escargots aux végétaux seuls (avec une grille surélevée laissant passer la végétation mais empêchant tout contact avec le sol), au sol seul (en ôtant toute la végétation au sein du microcosme) et aux deux sources (sol + végétation). Ce protocole pourra être répété pour plusieurs cortèges floristiques afin de mettre en évidence l'influence des types de végétaux. En effet, les contributions pourraient varier en fonction de la capacité des végétaux à accumuler les métaux ou en fonction de l'appétence de ces végétaux pour l'escargot.

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Partie F : ANNEXES

ANNEXE 1

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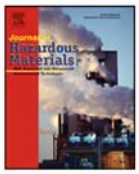

Use of chemical methods to assess Cd and Pb bioavailability to the snail *Cantareus aspersus*: A first attempt taking into account soil characteristics

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ABSTRACT

Bioavailability is a key parameter in conditioning contaminant transfer to biota. However, in risk assessment of terrestrial contamination, insufficient attention is being paid to the influence of soil type on trace metal bioavailability. This paper addresses the influence of soil properties on the chemical availability of cadmium (Cd) and lead (Pb) ($CaCl_2$ extraction and ionic activity) and bioavailability (accumulation kinetics) to the land snail *Cantareus aspersus*. Snails were exposed to nine contaminated soils differing by a single characteristic (pH or organic matter content or clay content) for 28 days. Toxicokinetic models were applied to determine metal uptake and excretion rates in snails and multivariate regression was used to relate uptake parameters to soil properties. The results showed that alkalinisation of soil and an increase of the organic matter content decreased Pb and Cd bioavailability to snails whereas kaolin clay had no significant influence. The $CaCl_2$-extractable concentrations tended to overestimate the effects of pH when used to explain metal uptake rate. We conclude that factors other than those controlling the extractable fraction affect metal bioavailability to snails, confirming the requirement of biota measurements in risk assessment procedures.

1. Introduction

In Europe, potentially polluting activities are estimated to have occurred at nearly three million sites [1]. This pollution, and other causes of soil quality degradation, led to the proposal of a thematic strategy for soil protection [2] which identifies pollution as the third threat of soil degradation. Trace metal elements represent the main contaminants (37.3%) among those affecting soil and groundwater quality in Europe [1] and constitute a great concern as they may cause environmental and public health problems [3]. In order to reduce these risks, protective concentration thresholds have been determined [4]. However, they are based on total soil concentrations and do not take into account the soil type although differences of physico-chemical properties are known to strongly influence metal mobility in soils [5] and their transfer to organisms [6–8]. Transfer corresponds to the passage of pollutants from one compartment (biotic or abiotic) to another. It is conditioned by both the exposure (i.e., the intensity of the contact with the contaminated environmental compartments) and the bioavailability of the trace metal with respect to a particular biological target [9]. Bioavailability is the proportion of the total concentration of a pollutant in soil which is available or has been made available in a dynamic manner over time to an organism from its direct environment. As a composite concept depending on many physical, chemical and biological issues, the bioavailability of contaminants can be assessed by the measurement of (i) environmental availability (usually by chemical extractions), (ii) environmental bioavailability (e.g. by analysis of absorption and assimilation of the pollutant by the organism) and (iii) toxicological bioavailability (by evaluating metal distribution to sites of action and the resulting physiological responses).

Pollutant uptake and accumulation are considered as good indicators of environmental bioavailability [6,7,10]. It is assumed that environmental bioavailability can be approached by measuring the internal concentration of a contaminant after a given exposure duration. However, this view is incomplete especially because it does not consider the dynamic aspects of bioavailability and bioaccumulation (time factor, equilibria, fluxes, etc.) [11]. For this purpose, one approach consists in describing the time course of trace metal concentration in an organism exposed to a contaminated medium by means of a one-compartment model [6,12]. Using modelled assimilation and excretion rates, the kinetics experiments focus on three essential aspects of risk characterization: bioavailability (assimilation flux) [6,13], bioaccumulation (steady-state internal concentration) and transfer (steady-state bioaccumulation factor)[14]. However, for an overall assessment of bioavailability, linking these biological tools with the physico-chemical processes driving the fate of metals in soil is recommended [15–17]. In this aim, the consideration of soil types

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Table 1
Properties of the three modified soils used in this study on which snails were exposed (soil C; control; K = soil C + clay (kaolin); P = soil C + peat; the associated number refers to the nominal soil pH value).

Soil	Soil characteristics		
	pH_{water}	Organic matter %	Clay %
5C	4.75	1.42	12.7
7C	7.10	1.38	12.8
8C	7.49	1.32	12.2
5K	4.56	1.30	16.3
7K	7.09	1.26	16.4
8K	7.46	1.20	15.2
5P	5.61	9.96	12.5
7P	6.95	8.10	12.2
8P	7.31	6.09	10.9

in risk assessment procedures appears to be of great value for the assessment of metals (environmental and toxicological) bioavailability to soil invertebrates [18,19].

Among the soil-dwelling invertebrates, the garden snail (*Cantareus aspersus*), as a recognised soil contamination bioindicator [20], is a good candidate to study metals bioavailability. Indeed, Gimbert et al. [6,21] pointed out that soil properties influence metal accumulation in the snail, but their experiments were not specifically designed to quantify the effect of soil characteristics on metal bioavailability to the snail. Therefore, the present study aimed, on the basis of an experimental design using spiked and artificially modified soil, to assess the influence of soil parameters (pH, organic matter (OM) and clay content) (i) on the environmental availability as evaluated by three chemical methods estimating different pools of metals in the soil and (ii) on the bioavailability of cadmium (Cd) and lead (Pb) to the snail *C. aspersus*, using kinetic model of accumulation. Final aim was (iii) to estimate if these chemical methods are suitable to assess metal bioavailability to snails using multivariate regression equations.

2. Materials and methods

2.1. Animals

Juvenile brown garden snails (*C. aspersus aspersus* Müller, 1774, syn. *Helix aspersa aspersa*) were reared as described by Gomot de Vaufleury and ISO [22,23] under controlled conditions. The individuals used for the test were subadults, reared for 7–9 weeks and weighing 4.88 ± 0.63 g (±SD; $n = 180$).

2.2. Soils

An uncontaminated agricultural field soil (Chambornay-les-Pins, Eastern France) (soil 5C) was used for further soil parameter modifications (Table 1). It was collected from the top 15 cm layer after removal of vegetation (grass) and transferred to the laboratory to be air-dried and, then sieved through a 4 mm mesh.

Soil characteristics (Table 1) were artificially modified by adding: (i) kaolin [24] to increase clay content (soil K) from 12% to 16%; (ii) dried ground peat [25] to raise organic matter content (soil P) from 1.4% to 8%; and (iii) $CaCO_3$ powder to obtain three nominal classes of pH (5, 7 and 8) [25,26].

The soils were spiked with Cd ($CdCl_2$, 99.9% purity, Aldrich Chemical) and Pb ($PbSO_4$, 99.9% purity, Aldrich Chemical) to reach nominal concentrations of 20 and 2000 mg kg^{-1} soil DW (Dry Weight), respectively. Deionised water was added to reach 50% of the respective water holding capacities of the different substrates. The soils were left to stabilize in the experimental conditions described below for one month before snail exposure.

2.3. Exposure

For each treatment, four snails were housed in each of five replicate polystyrene containers (24 cm × 21 cm × 8 cm) (ref. EIDB-BAC001, Charles River IFFA-CREDO, L'Arbresle, France) containing a 1-cm layer (100 g dry mass) of soil prepared as previously described. The snails were fed *ad libitum* with uncontaminated (0.7 ± 0.3 mg Cd kg^{-1} DW ($n = 3$) and 8.3 ± 0.7 mg Pb kg^{-1} DW ($n = 3$)) pieces of fresh lettuce put in a Petri dish. Therefore, the only source of contamination was the soil by both dermal and digestive routes [27,28]. The photoperiod was 18L/6D and the temperature was 20 ± 2 °C. The relative humidity was kept at 80–95%. Soil moisture content was maintained at its initial level by regular spraying with demineralised water. Three times a week, the containers were cleaned, any remaining food was renewed and demineralised water was sprayed to prevent drying out of soil.

After 0, 7, 14, 21 and 28 days, for each mode of treatment, one snail was randomly sampled from each of the five replicate containers.

2.4. Analytical procedures

2.4.1. Soils

Three chemical methods were investigated for the estimation of environmental availability: (i) total metal concentration, (ii) the $CaCl_2$ extractable concentration corresponding to the weakly bound metal in the soil and (iii) the total dissolved metal concentration in the soil solution. These methods have been chosen according to their capacity to extract the metal in different soil fractions and are commonly used in bioavailability assessment [18,29].

Total Cd and Pb measurements were made by inductively coupled plasma atomic emission spectrophotometry (ICP-AES) after digestion of the soil samples (250 mg) with hydrofluoric and perchloric acid and as described in [30].

$CaCl_2$ extracts were obtained after shaking 100 mL of 0.01 M $CaCl_2$ solution and 10 g of soil in an end-over-end shaker for 2 h at 30 rpm [29]. The supernatant was then centrifuged at 5000 rpm for 10 min and acidified (5%, HNO_3, Carlo-Erba analytical quality) for graphite furnace atomic absorption spectrometry (GFAAS) (Varian 220Z with Zeeman background correction) analysis.

Total dissolved metal concentration was calculated according to the method of Sauvé et al. [31] which estimates metal dissolved in the soil solution as a function of total soil metal concentration, pH for Pb and also the OM content for Cd.

2.4.2. Animals

Snails for analysis were placed in clean containers and fasted for 48 h (the faeces were removed after 24 h) then weighed. The snails were sacrificed by freezing (at −80 °C) before analysis. After thawing, the whole soft body was removed from the shell and the foot and the viscera were separated [32]. Only viscera were studied in this work because they contain the hepatopancreas (digestive gland) which is the main site of metal accumulation and storage in snails [33]. The viscera were oven-dried at 60 °C till constant weight (0.24 ± 0.05 g ($n = 180$)), digested in nitric acid (HNO_3 65%, Carlo-Erba analytical quality) and analysed by GFAAS, as previously described [32]. The validity of the analytical methods used was checked by analysing standard biological reference material (TORT-2, lobster hepatopancreas; National Research Council of Canada–Institute for National Measurement Standard, Ottawa, ON, Canada). The average deviations from the certified values were 2.1 ± 1.8% and 6.9 ± 3.3% for Cd and Pb, respectively.

Table 2
Total (mg kg^{-1}), 0.01 M $CaCl_2$-extractable (mg kg^{-1}; ±SD, $n = 3$) and estimated total dissolved (μg L^{-1}; calculated using the equations of Sauvé et al. [31]) concentrations of Cd and Pb in the treated soils. Similar letter shows absence of significant difference between the values.

Soils	Cd concentrations			Pb concentrations		
	Total mg kg^{-1}	$CaCl_2$ mg kg^{-1}	Dissolved μg L^{-1}	Total mg kg^{-1}	$CaCl_2$ mg kg^{-1}	Dissolved μg L^{-1}
5C	17.9	5.36 ± 0.12 ^{a}	261	1960	51.17 ± 6.90 ^{a}	78.7
7C	18.3	0.35 ± 0.16 ^{b}	21.5	1960	6.16 ± 5.19 ^{bc}	10.6
8C	15.7	0.12 ± 0.03 ^{c}	12.4	1740	4.12 ± 0.81 ^{c}	6.90
5K	17.9	5.66 ± 0.03 ^{a}	344	1910	79.32 ± 1.79 ^{d}	91.2
7K	18.4	0.41 ± 0.02 ^{b}	23.6	2000	4.17 ± 2.06 ^{bc}	10.8
8K	16.4	0.18 ± 0.01 ^{f}	14.5	1780	3.86 ± 1.05 ^{c}	7.40
5P	20.5	1.33 ± 0.14 ^{d}	24.6	2060	12.71 ± 1.11 ^{e}	38.9
7P	18.0	0.24 ± 0.01 ^{f}	5.90	1970	6,63 ± 0.79 ^{b}	12.1
8P	16.5	0.17 ± 0.03 ^{f}	4.60	1780	5.94 ± 2.83 ^{c}	8.40

2.4.3. Statistical analyses

Modelling. Bioavailability results from a dynamic interaction between the metal concentration in the soil (environmental availability) and the physiology of the target species. For its assessment, a one-compartment model was used to fit the accumulation kinetic data [6,7]. This model expresses the dynamic change of tissue metal concentration over time, following the equation (Eq. (1)):

$$C_{sn}(d) = C_{sn}(0) + \frac{a}{k_2}(1 - e^{-k_2 t}) \quad (1)$$

where the bioavailability of metal for snails is represented by the a = assimilation flux constant (mg_{metal} kg DWt_{sn}^{-1} d^{-1}); t = time (days); C_{sn} = metal concentration in the snail (viscera) (mg kg^{-1} snail dry weight); k_2 = the excretion rate constant (d^{-1}). $C_{sn}(0)$ is the average metal concentration measured in ten snails at the beginning of the experiment.

The steady state concentration $C_{sn}(ss)$ was used to characterize metal accumulation in snails as (Eq. (2)):

$$C_{sn}(ss) = C_{sn}(0) + \frac{a}{k_2} \quad (2)$$

Finally, the transfer of metal from soil to snail was characterized by calculation of the BAF (bioaccumulation factor) at steady-state using the kinetic parameters according to equation (Eq. (3)):

$$BAF_{(x)} = \frac{C_{sn}(ss) - C_{sn}(0)}{C_x} = \frac{k_{1(x)}}{k_2} \quad (3)$$

where (x) refers to the chemical method used and k_1 = uptake rate constant (see below).

The accumulation and elimination parameters were estimated by fitting the models with a mixed-effects procedure (nlme, [34]) allowing for nested random effects. The within-group errors were allowed to be correlated and/or have unequal variances. The nlme integrated the soil as a fixed factor and the container as a random effect. Statistical differences in parameter estimates between treatments were judged from the absence of overlap of their 95% confidence intervals (95% CI). To validate the accumulation kinetics, a Wilcoxon test was performed between the modelled and the measured accumulation after 28 days exposure.

2.5. Relating uptake to soil characteristics

The efficiency of an extractant to assess bioavailability (represented by the assimilation flux, a) was checked by deriving an uptake rate constant (k_1; represents the exposure of the snail to the metals) allowing the influence of soil parameters to be compared with both bioavailability (a) and environmental availability (C_x) (Eq. (4)):

$$k_{1(x)} = \frac{a}{C_x} \quad (4)$$

Insofar as the assimilation flux (a) is constant over the duration of exposure, the units in which the uptake rate (k_1) is expressed depends on the units of the hypothesised bioavailable metal concentration (C_x) estimated according to (i) the total metal concentration in the soil (C_{tot} in mg kg DWt_{soil}^{-1}), (ii) the $CaCl_2$-extractable metal concentration (C_{CaCl_2} in mg kg DWt_{soil}^{-1}) and (iii) the total dissolved metal (C_{pw} in μg L^{-1}). The unit of k_1 is express in g_{soil} g DWt_{sn}^{-1} d^{-1} (for total metal concentration or $CaCl_2$ extraction) or in L kg DWt_{sn}^{-1} d^{-1} (for total dissolved metal in the soil solution).

The variation of uptake rates and BAFs among soils was expressed as a multivariate function of the measured soil characteristics (Eq. (5)):

$$\log(Y) = x\log(A) + y\log(B) + \ldots, z \quad (5)$$

where $Y = k_{1(x)}$ or $BAF_{(x)}$; x, y,... represent the coefficients and A, B,... represent the soil parameters [7]. Only descriptors with p-value <0.05 were included in the model.

In order to relate environmental availability (C_x) to bioavailability (assimilation flux, a) taking into account the influence of soil properties, the chemical method has to simulate the influences of both soil characteristics and physiology on bioavailability. To do so, we used the uptake rate ($k_1 = a/C_x$) which clearly takes these two contributions into account in its estimation but which not directly represents the bioavailability. We assumed that when the soil properties influenced environmental availability (C_x) and bioavailability (a) identically, the regression of uptake rate (k_1) estimate against soil properties will not be significant. In this case, the chemical extractable concentration in soil (C_x) can be used to assess the bioavailability (a) of the metal for the snails. When the regression was significant, the chemical extractant cannot be used to simulate the bioavailability because of the higher or lower influence of the soil properties (pH, OM and clay contents) on environmental availability (C_x) than on bioavailability (a). These over- or underestimations will result to positive or negative values of the parameter coefficients, respectively in the regression equations. The determination coefficients (r^2) of each regression were used to determine whether the main soil parameters influencing bioavailability were included in the model (high r^2) or if other parameters should be taken into account (low r^2).

All statistical analyses were performed with the free statistics software package R 2.6.0) [35].

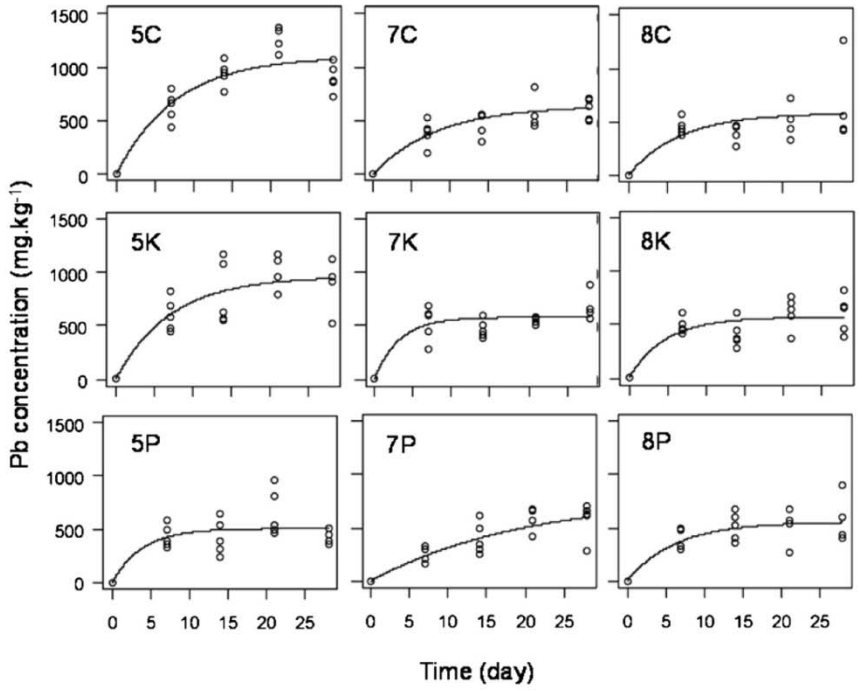

Fig. 2. Modelled accumulation kinetics of Pb in *Cantareus aspersus* snails exposed to the soils C, soils K (i.e. soil C + clay) and soils P (i.e. soil C + organic matter) with increasing pH. The Cd concentrations in snail's viscera are expressed on a dry weight basis. The curve represents the accumulation kinetic. Each data point represents an individual snail.

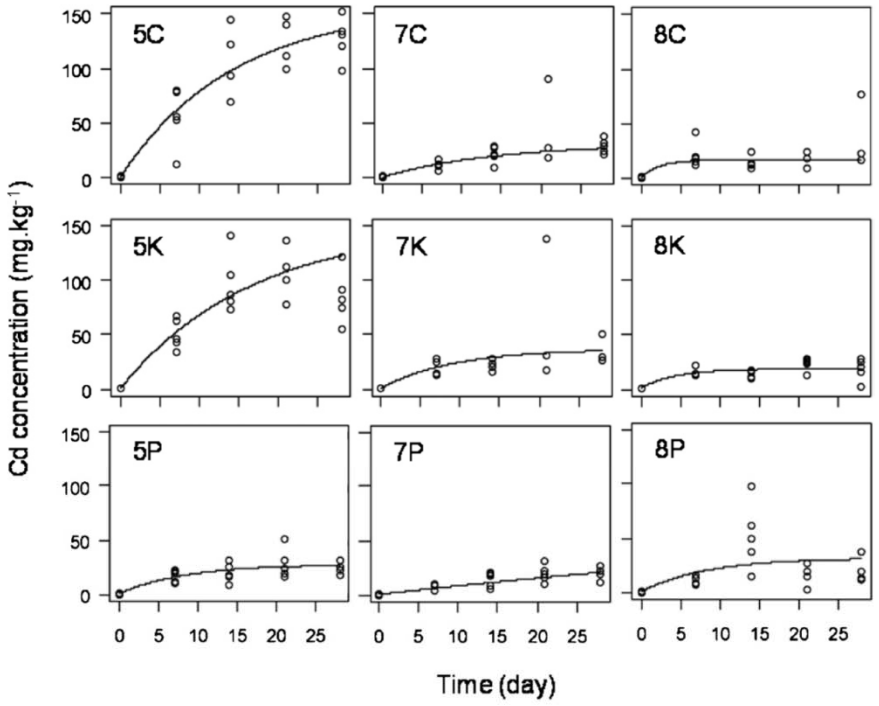

Fig. 1. Modelled accumulation kinetics of Cd in *Cantareus aspersus* snails exposed to soils C, soils K (i.e. soil C + clay) and soils P (i.e. soil C + organic matter) with increasing pH. The Cd concentrations in snail's viscera are expressed on a dry weight basis. The curve represents the accumulation kinetic. Each data point represents an individual snail.

Table 3
Estimates of kinetic parameters for Cd and Pb accumulation in *Cantareus aspersus* snails exposed to the contaminated soils. (a) corresponds to the assimilation flux reflecting bioavailability, k_2 corresponds to the excretion rate, $C_{sn}(28)$ corresponds to the mean (±SD) of snails viscera concentration after 28 days of exposure, $C_{sn}(ss)$ corresponds to the steady state in the snails viscera. na: absence of internal steady state concentration due to absence of metal excretion.

Soils	Measured	Modelled						r^2	Calculated	
	$C_{sn}(28) \pm SD$ ($n=5$) $mg_{metal}\ kg^{-1}$	$a\ (k_1\ C_x)$ $mg_{metal}\ kg_{sn}^{-1}\ d^{-1}$	95% CI	p-value	k_2 d^{-1}	95% CI	p-value		$C_{sn}(28)$ $mg\ kg^{-1}$	$C_{sn}(ss)$ $mg\ kg^{-1}$
Cadmium										
5C	127 ± 19.8	10.9 [a]	9.01/12.8	<0.001	0.07 [a]	0.04/0.09	0.020	0.84	117	159
7C	29.0 ± 6.29	2.12 [b]	1.71/2.52	<0.001	0.07 [a]	0.04/0.09	0.038	0.99	27.9	32
8C	33.3 ± 28.8	6.77 [abc]	0.23/13.1	0.370	0.41 [ab]	−0.00/0.83	0.390	0.94	16.9	na
5K	84.7 ± 24.5	8.95 [a]	7.82/10.1	<0.001	0.06 [a]	0.04/0.09	0.045	0.71	89.9	140
7K	33.8 ± 11.0	3.95 [c]	2.76/5.13	0.010	0.11 [ab]	0.03/0.18	0.230	0.29	36.4	na
8K	17.9 ± 10.4	4.11 [c]	2.58/5.65	0.027	0.23 [b]	0.13/0.34	0.610	0.57	18.4	na
5P	26.1 ± 5.62	3.24 [b]	2.32/4.15	0.006	0.12 [ab]	0.07/0.17	0.040	0.62	26.9	27
7P	21.4 ± 5.57	0.83 [c]	0.60/1.06	0.005	−0.00 [ab]	−0.03/1.06	0.240	0.76	26.1	na
8P	20.4 ± 1.78	3.73 [b]	1.15/6.31	0.086	0.12 [ab]	−0.00/0.25	0.260	0.22	29.9	na
Lead										
5C	900 ± 132	142 [a]	121/164	<0.001	0.13 [a]	0.10/0.16	<0.001	0.86	1071	1101
7C	612 ± 99.1	76.4 [b]	59.9/93.0	<0.001	0.12 [a]	0.08/0.15	<0.001	0.90	620	639
8C	671 ± 402	87.2 [bc]	58.8/115	0.015	0.15 [a]	0.06/0.23	0.130	0.82	580	na
5K	892 ± 221	134 [ac]	106/161	<0.001	0.14 [a]	0.10/0.18	0.006	0.79	940	957
7K	678 ± 139	178 [ac]	102/254	0.053	0.31 [a]	0.15/0.46	0.074	0.80	567	na
8K	596 ± 175	119 [abc]	82.1/155	0.010	0.21 [a]	0.14/0.28	0.020	0.73	564	567
5P	419 ± 57.6	131 [abc]	88.5/173	0.013	0.26 [a]	0.15/0.36	0.039	0.64	504	505
7P	576 ± 164	39.6 [d]	33.1/46.0	<0.001	0.05 [b]	0.03/0.07	0.047	0.80	602	793
8P	587 ± 225	88.1 [abc]	71.7/105	<0.001	0.16 [a]	0.10/0.21	0.021	0.74	560	553

95% CI values are likelihood-based 95% confidence intervals. For one parameter for a given metal, values that share similar letters are not significantly different. Negative values of the excretion rates (k_2) should be biologically considered as an absence of excretion. Significant differences between parameters were assessed by absence of overlapping of their 95% CI.

3. Results

3.1. Environmental availability

The soil characteristics are summarised in Table 1. At the beginning of the experiment, three distinct classes of pH were chosen; an indication of the pH is given by the numerical values in the soil name. Differences between nominal and actual soil pH values, especially for alkaline pH, were due to the difficulty of adjusting the pH using only $CaCO_3$ without excessively disrupting other soil characteristics (especially OM and clay contents).

Cadmium and Pb concentrations in the different pools studied are presented in Table 2. For the total soil concentration and estimation of the total dissolved metal concentration, only one replicate was available for each soil, thus we were unable to carry out statistical analyses. Total metal concentrations in soil did not show any specific trends of variation following the soil parameter modifications. However, pH did have an impact on total dissolved metal concentrations: a soil pH increasing from 5 to 7, caused a decrease in total dissolved metal concentrations from 76% to 93% for Cd and from 69% to 89% for Pb (Table 2). Whatever the soil pH, an increase in OM content led to a decrease in estimated total dissolved Cd concentrations in the soil solution while an increase in clay content did not affect dissolved Cd concentrations. For Pb, the decrease observed from 78.7 to 38.9 $\mu g\ L^{-1}$ was due to the difference in pH (soil 5C: pH 4.7; soil 5P: pH 5.6), indeed the OM content was not taken into account during total Pb concentration in soil solution calculation. Calcium chloride solution (0.01 M $CaCl_2$) extracted from 1% to 32% total Cd and from 0.3% to 4% total Pb (Table 2). With an increase of two pH units a significant decrease of extractable metals was found: between 96% and 99% for Cd and between 46% and 95% for Pb ($p < 0.001$). Calcium chloride-extracted Cd concentrations decreased by 2% to 26% with an increase of the OM content ($p < 0.001$). However, for Pb the impact of OM addition decreased $CaCl_2$ extractable concentrations only in the most acidic soil (soil 5P, Table 2). In most cases, an increase of the clay content led to non-significant increases of the environmental availability.

3.2. Environmental bioavailability

3.2.1. Survival and growth

Snail mortality was low during the experiment, reaching 7% at the end of the exposure period (28 d) and similar in the different soils (from 4% in soil P to 9% in soil C).

During exposure, the fresh mass of the snails did not change significantly (final mass: 5.0 ± 0.6 g versus 4.9 ± 0.6 g for the initial mass, $p = 0.17$, $n = 180$). Therefore, growth was not considered in the accumulation kinetics model as a potential dilution factor [6].

3.2.2. Accumulation kinetics

At the beginning of exposure, the internal concentrations in snails were 0.84 ± 0.41 mg kg DWt^{-1} for Cd and 2.77 ± 0.80 mg kg DWt^{-1} for Pb ($n = 10$).

The toxicokinetic model adequately described the time course of Cd and Pb concentrations as shown by the determination coefficient (r^2) and the non-significant differences between calculated and modelled internal concentrations after 28 days of exposure ($p = 0.77$) (Table 3).

Two characteristic non-linear patterns of accumulation were distinguished: many Cd accumulation patterns did not reach a plateau underlining the absence of a steady state internal concentration (Fig. 1; Table 3). This difference should be viewed with respect to the low and often non-significant Cd excretion over the exposure duration (Table 3), preventing internal Cd concentrations from reaching a steady-state. Only four of the nine soils led to Cd excretion rates that were significant, and always when the pH was low. In this case, accumulation tended to stabilize after 15–21 days of exposure (Fig. 1).

For Pb, fast assimilation coupled with significant excretion rates led a steady-state in internal concentrations, usually after 15 days of exposure to the different soils (Table 3; Fig. 2). Only snails exposed to the soils 8C and 7K showed no significant excretion (k_2) ($p = 0.130$ and 0.074) (Table 3), and therefore the steady state concentrations could not be calculated.

Table 4
Multivariate regression formulae relating the uptake rate (k_1) and bioaccumulation factor (BAF) to soil characteristic using different chemical method of environmental availability estimation (total soil concentration, $CaCl_2$ extractable and total dissolved concentration) for Cd and Pb for *Cantareus aspersus* exposed on contaminated soil. na: absence of regression due to the absence of BAF value. ±Standard error (SE).

Metal	Chemical method	Type of parameter	Equation	r^2_{adj}	*p*-value
Cd	Total soil concentration	Kinetic	$\log k_1\,(\text{total}) = -0.21 \pm 0.06 \cdot \text{pH}^{*} - 0.60 \pm 0.18 \cdot \log \text{OM}^{*}$	0.77	0.023
		Steady state	$\log \text{BAF}\,(\text{total}) = -$	na	na
	$CaCl_2$ extraction	Kinetic	$\log k_1\,(CaCl_2) = 0.29 \pm 0.08 \cdot \text{pH}^{*} - 1.56 \pm 0.61^{*}$	0.70	0.012
		Steady state	$\log \text{BAF}\,(CaCl_2) = -$	na	na
	Total dissolved metal	Kinetic	$\log k_1\,(\text{diss.}) = 0.26 \pm 0.06 \cdot \text{pH}^{**} - 2.62 \pm 0.40^{**}$	0.73	0.009
		Steady state	$\log \text{BAF}\,(\text{diss.}) = -$	na	na
Pb	Total soil concentration	Kinetic	$\log k_1\,(\text{total}) = -$	0.09	0.240
		Steady state	$\log \text{BAF}\,(\text{total}) = -$	0.21	0.163
	$CaCl_2$ extraction	Kinetic	$\log k_1\,(CaCl_2) = 0.31 \pm 0.07 \cdot \text{pH}^{**}$	0.74	0.004
		Steady state	$\log \text{BAF}\,(CaCl_2) = 0.33 \pm 0.03 \cdot \text{pH}^{***}$	0.94	<0.001
	Total dissolved metal	Kinetic	$\log k_1\,(\text{diss.}) = 0.29 \pm 0.05 \cdot \text{pH}^{**} - 1.16 \pm 0.34^{*}$	0.81	0.001
		Steady state	$\log \text{BAF}\,(\text{diss.}) = 0.31 \pm 0.04 \cdot \text{pH}^{***}$	0.92	<0.001

* Statistical significance for $p < 0.05$.
** Statistical significance for $p < 0.01$.
*** Statistical significance for $p < 0.001$.

Concerning comparison of the excretion rates, significant k_2 values were systematically lower for Cd than for Pb (Table 3). Globally, neither Cd nor Pb excretion rates showed significant differences between soil treatments.

In all treatments, assimilation of Cd was lower than that of Pb (Table 3). Indeed, the increase of Cd and Pb concentrations in the viscera (Figs. 1 and 2) was reflected by the assimilation fluxes ranging from 0.830 to 10.9 $mg_{metal}\,kg_{snail}^{-1}\,d^{-1}$ and 39.6 to 177 $mg_{metal}\,kg_{snail}^{-1}\,d^{-1}$ for Cd and Pb, respectively (Table 3). An increase of two pH units was associated with the assimilation fluxes decreasing from 10.9 to 2.12 $mg_{metal}\,kg_{snail}^{-1}\,d^{-1}$ for Cd and from 143 to 76.4 $mg_{metal}\,kg_{snail}^{-1}\,d^{-1}$ for Pb (Table 3). However, the assimilation flux of Cd was not significant in two modalities with the highest pH (8C and 8P, Table 3). At the same time, an 8% increase of OM content caused assimilation fluxes to fall from 10.9 to 3.24 $mg_{metal}\,kg_{snail}^{-1}\,d^{-1}$ for Cd (pH 5) and from 76.4 to 39.6 $mg_{metal}\,kg_{snail}^{-1}\,d^{-1}$ for Pb (pH 7). The increase in clay content led to an increase of the assimilation fluxes in the soil with a neutral pH. Most of the time, no difference was observed between the pH 7 and pH 8 treatments due to the small difference in actual pH observed between these soils (Table 1). Taking into account the different estimates of environmental availability, uptake rates (k_1) derived showed large differences between soil treatments. For Cd, they rose from 0.050 to 0.600 $g_{soil}\,g_{sn}^{-1}\,d^{-1}$ on the basis of total soil concentrations, from 1.57 to 67.70 $g_{soil}\,g_{sn}^{-1}\,d^{-1}$ based on $CaCl_2$ extractable concentrations and from 0.02 to 0.81 L kg $DWt_{sn}^{-1}\,d^{-1}$ for total dissolved metal concentrations. Similar differences were found for Pb uptake rates.

3.3. Multivariate analyses relating uptake characteristics and soil properties

Table 4 presents the results of the multivariate regressions and the soil parameters that most explained Cd and Pb uptake rate (k_1) and Pb transfer to the snails (BAF). We were unable to estimate the influence of soil properties on Cd BAF due to the small number of steady-state BAF values. Two non-significant regressions were identified, both considering the total soil concentration as the hypothesised bioavailable Pb pool. Other regressions considering $CaCl_2$ extractable and dissolved metals as bioavailable were significant and presented variable but elevated determination coefficients. As observed for environmental availability, soil pH and OM content influenced metals uptake rate and transfer while the clay content was not identified as a significant variable (Table 4). The coefficient of OM content was negative (underestimation) whereas pH coefficients presented both negative and positive signs. All the positive coefficients (overestimation) were found for k_1 or BAF derived using $CaCl_2$ extractable or total dissolved metals; the only negative coefficient was obtained for the k_1 estimation based on total Cd soil concentration.

4. Discussion

4.1. Bioavailability of Cd and Pb to C. aspersus and influence of soil characteristics

Comparison of the assimilation fluxes for Cd and Pb between the different soils showed a clear influence of soil properties on the bioavailability of soil trace metals to snails.

These two metals were selected for their differences in accumulation kinetics, as previously described by Gimbert et al. [36]. In most cases, Cd accumulation did not reach internal steady state concentrations during the 28 days of exposure, while Pb did. This was due to different excretion rates related to two different sequestration strategies carried out by snails [33,36–38]. Moreover, for both metals, the excretion rates tended to be significant for the high assimilation fluxes in soils with the lowest pH. Indeed, an increase of bioavailability caused by soil acidification or by a decrease of OM content was shown. This is in accordance with the higher extractable concentration of Cd and Pb resulting from a decrease in pH. Previous studies [26,39] have shown that Cd and Pb speciation depended partly on soil acidity. Indeed alkaline soils might modify colloid effects on proton activity, decreasing their solubility. Sauvé et al. [31] reported that Pb and Cd partitioning coefficients were explained to 47% by the pH. Concerning the modification of OM content, our results are in accordance with those of Hooda and Alloway [40] who demonstrated that a soil with a high OM content has high metallic adsorption capacities. Indeed, OM has the ability to adsorb Cd and Pb specifically by association to –OH groups (e.g. covalent bonds) and it has been shown on forest soils, that 23–47% of the anthropogenic Pb was bound to OM [41]. Therefore, an increase in the number of binding sites is expected to lead to a modification of environmental availability and potentially, bioavailability. This was confirmed by our results using snails which have different sources of contamination (soil, plant, etc.) [27,28], proving the importance of soil parameters on bioavailability. Van Gestel and Koolhaas [26] found similar results with a collembolan (*Folsomia candida*). However, the difference of metal sources between this

hard bodied soil dwelling arthropod and a soft bodied soil invertebrate, such as a snail, has been underlined by Schipper et al. [42]. Soft bodied organisms are more exposed to the metal coming from the soil pore water by dermal contact than hard bodied organisms. For both metals, an increase of clay content in soil did not seem to influence the bioavailability (assimilation flux). It was hypothesised that metal transfer would decrease with an increase in the proportion of clay because Cd and Pb bind to it [43]. However, Madrid et al. [44] found that the metals present in the clay fraction are under a bioavailable form. The absence of clay effect on bioavailability in the present work can be due to the type of clay that we used. Indeed, kaolinite has a much lower metal binding capacity than montmorillonite [45].

4.2. Using chemical method to estimate the bioavailability

Nowadays numerous studies have proposed chemical methods to measure trace metal bioavailability [16,46–48], but none is suitable to predict bioavailability for all organisms [49]. Studying bioavailability using a living system coupled with chemical methods is promising to compensate for the lack of a consensus method [19,49].

The ability of snails to accumulate Cd and Pb is well documented in static [20,28] as well as in kinetic studies [6,21]. Some of these studies suggested that soil type could modify the transfer of metals from soil to snail [6,20]. Present data demonstrated that pH and OM content are essential to modulate the transfer and bioavailability of Cd and Pb from soils to snails. In some cases, multivariate expressions of BAF and uptake rate estimations as a function of soil properties have been established. It constitutes a first attempt to take into account soil properties for bioavailability assessment even for organisms which are not completely restricted to soil, like snails. The same influence of pH and OM have been observed in oligochaetes [7].

The use of total soil concentration to predict the variation of Cd and Pb bioavailability and transfer does not appear to be a reliable approach, as the influence of soil characteristic is ignored. That is why, for risk assessment purposes, combining chemical and biological measurements is encouraged [7,24,47,50]. It underlines the importance of species and particularly the influence of physiology during bioavailability assessment [51]. Concerning Pb, the absence of significant regression using the total soil concentration is due to the great deviation of assimilation flux and then uptake rate (Tables 3 and 4).

At the same time, for the two other chemical methods used, which take into account the influence of soil properties on bioavailability, all the variations of uptake rates or BAFs are explained by the soil parameters. The similarity between regressions using uptake rate or BAF for the same chemical method indicates the absence of an impact of soil parameter on excretion rate in the range of metal concentrations and soil parameters studied. Influences of soil parameters, particularly the pH, on metal bioavailability and transfer are not fully represented by the extractant. The pH seems to be an important parameter which influences both soil metal partitioning and animal physiology. This effect of pH has already been demonstrated during toxicity tests, showing that pH can have a protective effect on free metal ion toxicity [18,52]. Here, the effect of pH on environmental availability is probably well estimated by these chemical methods but its influence on snail physiology may be poorly estimated. That is why, for regression with $CaCl_2$ extractable and total dissolved metal concentrations, all pH coefficients had positive signs (except when considering the total concentration of Cd), signifying an overestimation of the influence of pH on metal bioavailability and transfer. This is due to the higher influence of pH on environmental availability than on bioavailability. The increase of two pH units has a greater influence on the C_x estimates using $CaCl_2$ (decrease of 90% and 76% for Cd and Pb respectively) than on the assimilation fluxes (decrease of 70% and 43%). Using the total dissolved concentration may, in some cases, lead to an overestimation of the pH effect during bioavailability and transfer assessment. Even though the main soil parameters (pH, OM and clay) which influence bioavailability [7,9,53] have been included in the model, as testified by the high determination coefficients, $CaCl_2$-extractable and total dissolved concentrations cannot be used to assess the bioavailability and transfer of metals from soil to the snails. Peijnenburg et al. [53] found that the Pb bioavailable concentration to enchytraeids was well related to $CaCl_2$ extraction. However, biology, exposure sources and routes for enchytraeids and snails are not identical (enchytraeids have a closer contact with the soil). These differences underline the importance of taking into account the sources of metal, the physiology of the test species and the soil parameters (like pH, OM content, ageing...) during bioavailability and transfer assessment. Moreover, these authors' interpretation of multivariate regression (i.e. the higher the r^2, the better the regression and the extractant) presented some differences with ours that could have led to different conclusions.

5. Conclusions

Risk assessment procedures have to take into account the species characteristics (including physiology, routes of contamination, etc.) and the soil parameters which modulate bioavailability and thus the risk of the organisms exposed. This study underlines the importance of considering soil parameters for assessing metal bioavailability and transfer to snails. The parameters that mainly influence the bioavailability (pH and OM) are the same as those that influence environmental availability as measured by chemical methods. However, $CaCl_2$-extractable metal or total metal dissolved in soil solution are not suitable to estimate the soil Cd and Pb fraction bioavailable to snails. The dynamic approach proposed in this work to select a suitable chemical extraction process to assess bioavailability is promising and should improve relating soil metal availability to bioavailability for *C. aspersus* snails. This methodology is usable with classical BAF (ratio of contaminant in snail to contaminant in environment) but calculated for effective steady state conditions, which are frequently hard to check without kinetic data. This difficulty is bypassed with our kinetic approach which allows to work with estimated steady state internal concentration and BAF, even if equilibrium is not reached over the exposure period.

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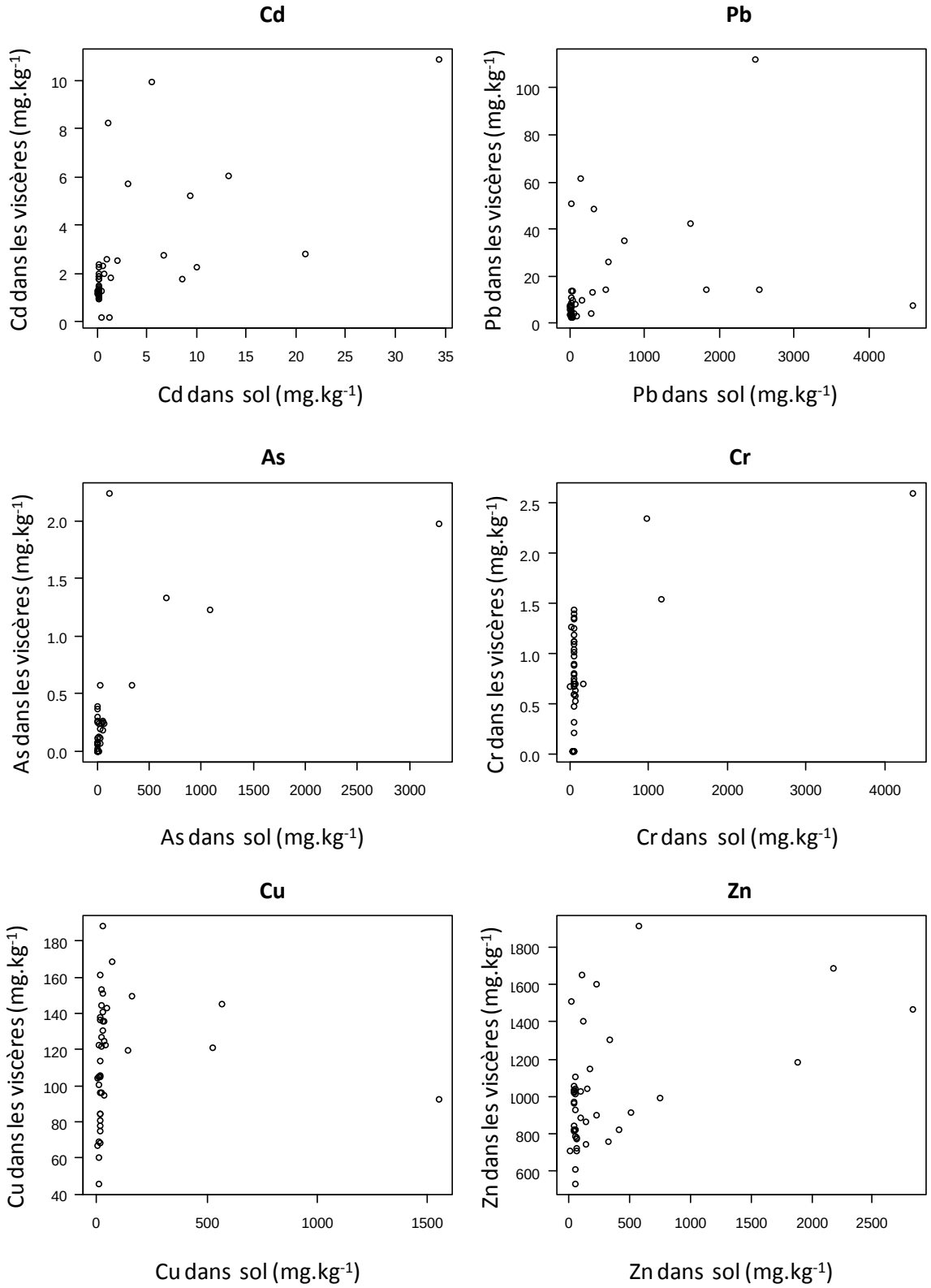
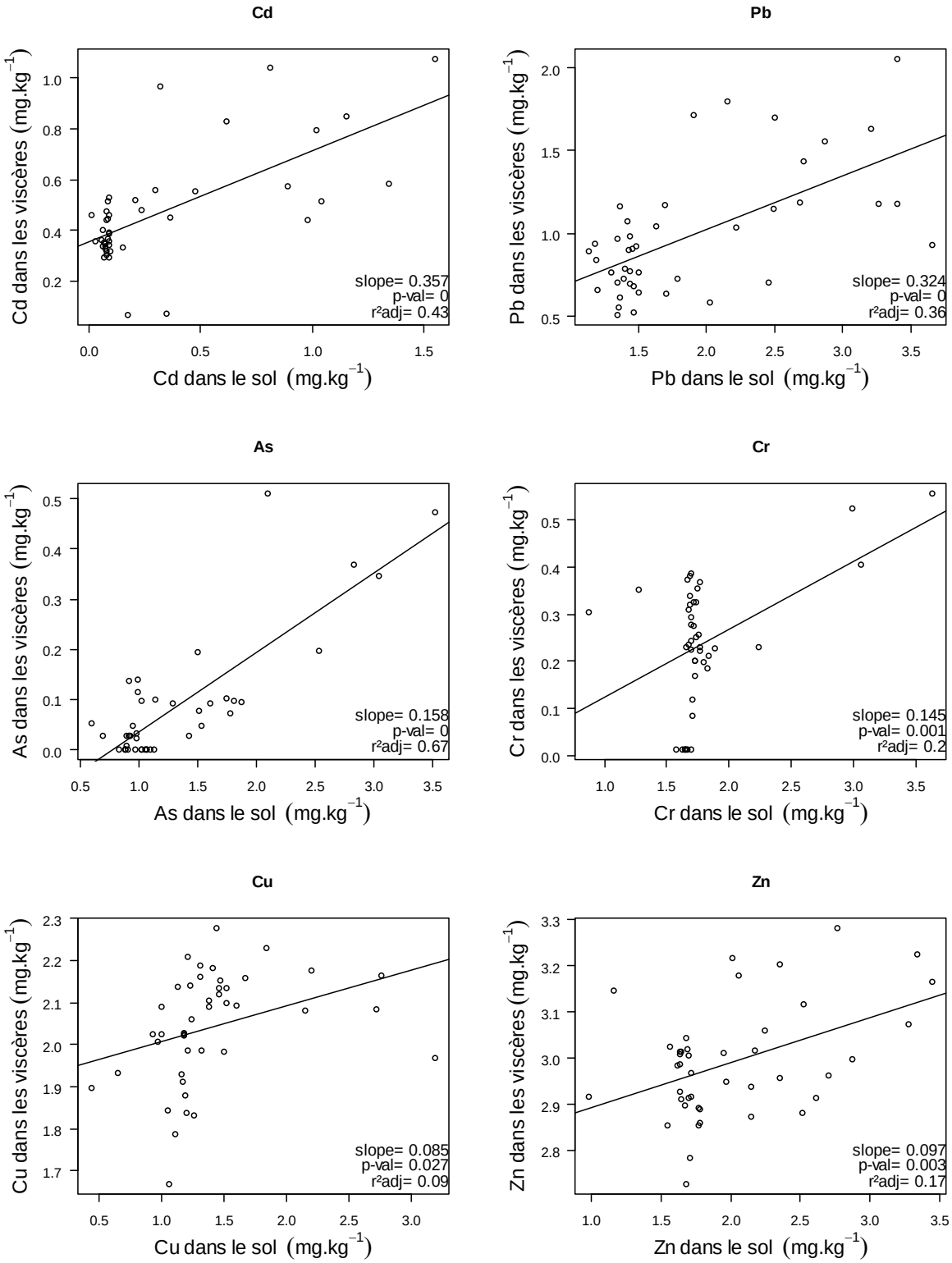
Annexe 2

Représentation des médianes des concentrations dans les viscères des escargots après 28 jours d'exposition en focntion des médianes des concentrations totales dans le sol en Cd, Pb, As, Cr, Cu et Zn.



Relations linéaires entre les log+1 des médianes des concentrations dans les viscères des escargots après 28 jours d'exposition et les log+1 des médianes des concentrations totales dans le sol en Cd, Pb, As, Cr, Cu et Zn.



Abstract:
Current environmental risk assessment (ERA) related to the soil contamination by metal consider neither the physico-chemical properties of soil nor the biological mechanisms that modulate metal bioavailability. The aim of this thesis is to study the mechanisms that modulate metal bioavailability for the garden snail *Cantareus aspersus* (= *Helix aspersa*) a soil invertebrate living at the interface soil-plant-air (saprophagous and phytophagous intermediate link in the food chain). Bioavailability is assessed by the metal accumulation (internal concentration of metals after 28 days of exposure) and assimilation fluxes. The influence of soil characteristics on availability and bioavailability of metal in soil and contribution of the contamination sources (soils/plants) of snails are the studied variables in two exposure protocols:

In situ, on the twelve sites of the ADEME bioindicator 2 program, multivariate equations have identified organic carbon (C_{org}), oxides (iron and aluminium) and CEC as modulating parameters of accumulation of Cd, Pb, Cr and Zn and assimilation of Cd and Pb. However, soil properties do not influence neither As assimilation and accumulation nor Sb accumulation. These studies highlight the low bioavailability of As and Sb in soil. Total metal concentration and characteristics of soil allow the explanation of 40 to 67% of accumulated metal in snails. The addition of other variable like plant contribution on snails contamination may improve the understanding of metal bioavailability modulation for snails. These results have led to the establishment of internal concentration of reference (CIRef) of snails exposed on uncontaminated site. Determined for As, Cd, Cr, Cu, Pb and Zn, these CIRef, compared to the internal concentration of snails exposed on various contaminated sites have permit the characterization of the variable bioavailability of metals. The proposed methodology of interpretation (SET: Sum of Excess of Transfer) may define environmental management priorities not based on total metal concentration in soil but on a biological risk assessment intregrating the soil properties influence on metal bioavailability to snails.

In laboratory, by using an agricultural field soil manually modified (contamination, pH and organic matter content) we have determined that the main assimilation source of Pb is the soil (90%) whereas the one of Cd is the lettuce (80%). For both meals, a decrease of two-pH-unit increases the lettuce contribution since an increase of organic matter content increases the soil contribution. By using 17 other soil coming from a contaminated site we identified pH, CEC and oxides (iron and aluminium) as modulating parameter of Cd, Pb and Zn bioavailability for snails. For these soils, total soil metal concentration and EDTA extractable fraction are usable to assess respectively Pb and Cd bioavailability for snails. It signifying that metals strongly bound on the solid phase of soil are bioavailable for snails. For Zn, none chemical method alone allows its bioavailability assessment, its assessment can be only done by using total metal concentration and properties of soil (pH and Al and Fe oxides).

These data underline the necessity to take into account the factors and mechanisms that modulate the metal bioavailability for snails to better model accumulation and assimilation of metal by snails. As no unique chemical method to assess metal bioavailability was determined, we recommend the use of biological measures that identify the real metal bioavailability rather than the use of chemical measures.

Keywords: snail, availability, bioavailability, metal, environmental risk assessment, accumulation kinetics, soil parameters, contamination sources

Résumé:

Les procédures réglementaires actuelles d'évaluation des risques environnementaux (ERE) des sols contaminés par les métaux ne considèrent ni les propriétés physico-chimiques des sols ni les mécanismes biologiques qui conditionnent pourtant la biodisponibilité des contaminants. L'objectif de cette thèse est l'étude des mécanismes modulant la biodisponibilité des métaux pour l'escargot *Cantareus aspersus* (syn. *Helix aspersa*), invertébré vivant à l'interface sol-plante-air (maillon intermédiaire, saprophage, phytophage, de chaînes alimentaires). La biodisponibilité est principalement évaluée ici en mesurant l'accumulation (concentrations internes en métaux après 28 jours d'exposition) et les flux d'assimilation. L'influence de paramètres édaphiques sur la disponibilité et la biodisponibilité des métaux des sols et la contribution des sources de contamination (sols/plantes) des escargots constituent les variables étudiées dans deux conditions d'exposition :

In situ, sur les douze sites du programme ADEME Bioindicateurs 2, nous avons identifié grâce aux régressions multivariées que le carbone organique (C_{org}), les oxydes (de fer et d'aluminium) et la CEC modulent l'accumulation du Cd, du Pb, du Cr et du Zn ainsi que l'assimilation du Cd, du Pb. Par contre, les paramètres des sols n'affectent ni l'accumulation et l'assimilation de l'As, ni l'accumulation du Sb. Ces études révèlent la faible biodisponibilité de l'As et du Sb dans les sols des sites étudiés. La concentration totale et les caractéristiques des sols permettent d'expliquer 40 à 67% de l'accumulation de métaux dans les escargots. L'intégration d'autres compartiments, notamment la végétation, pourrait améliorer la compréhension des variations de biodisponibilité pour les escargots. Ces résultats ont permis de proposer des concentrations internes de références (CIREF) d'escargots exposés sur les sites non contaminés. Déterminées pour As, Cd, Cr, Cu, Pb et Zn, ces CIREF, confrontées aux concentrations internes d'escargots exposés sur des sites diversement contaminés en métaux, ont permis de caractériser la biodisponibilité variable des métaux. La méthode d'interprétation proposée (SET : Somme des Excès de Transfert) peut ainsi aider à définir les priorités de gestion environnementale non plus seulement sur la base des concentrations totales en métal dans le sol mais sur une évaluation biologique des risques intégrant l'influence des propriétés du sol sur la biodisponibilité des métaux pour les escargots.

En laboratoire, à partir d'un sol agricole artificiellement modifié (contamination, pH et taux de matière organique) nous avons déterminé que la source principale d'assimilation du Pb est le sol (90%) tandis que celle du Cd est la laitue (80%). Pour ces 2 métaux, une diminution de deux unités de pH du sol augmente la contribution de la laitue, tandis qu'une augmentation du taux de matière organique du sol augmente la contribution du sol. A l'aide de 17 autres sols issus d'un site contaminé, nous avons observé que le pH, la CEC et les oxydes (Fe et Al) modulent la biodisponibilité du Cd, du Pb et du Zn pour les escargots. Pour ces sols, la concentration totale en métal du sol et la fraction extractible à l'EDTA sont utilisables pour évaluer respectivement la biodisponibilité du Pb et Cd. Les résultats obtenus suggèrent que les escargots ont accès à la fraction des métaux fortement liés à la phase solide du sol. Pour le Zn, aucune méthode chimique seule ne permet d'estimer sa biodisponibilité, cette dernière étant prédite seulement en associant la concentration totale et les caractéristiques du sol (pH et oxydes d'Al et de Fe).

L'ensemble des résultats souligne la nécessité de prendre en compte les facteurs et les mécanismes qui modulent la biodisponibilité des métaux pour modéliser au mieux leur accumulation et leur assimilation par les escargots. Aucune méthode chimique unique d'estimation de la biodisponibilité des métaux n'ayant pu être déterminée, nous préconisons l'utilisation de mesures biologiques qui reflètent mieux la réelle biodisponibilité.

Mots-clés: escargots, biodisponibilité, disponibilité, métal, évaluation des risques, cinétiques d'accumulation, paramètres du sol, sources de contamination.